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■ RESEARCH ARTICLE

Behavioral Effects of Non-Lethal Doses of Lead Nitrate on Fathead Minnows (*Pimephales promelas*)

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ABSTRACT: Non-lethal doses of lead compounds such as lead nitrate can negatively impact aquatic life without causing death. The extent of the impact of non-lethal doses of lead nitrate on fathead minnow (*Pimephales promelas*) behavior is unknown. A five-day acute toxicity test was executed and repeated five times to determine lead nitrate's effects on fathead minnow activity and respiration. Fathead minnows were exposed to various environmentally relevant concentrations of lead nitrate and observed for four full days following a 24-hour acclimation period. Preliminary findings found statistically significant evidence that exposure to lead nitrate corresponded to a decrease in activity frequency and a negative linear relationship between lead nitrate concentration and activity engagement. The findings of the present study suggest the need for EPA guideline re-evaluation as non-lethal doses can affect the behavior of aquatic organisms such as fathead minnows and inhibit their ability to survive.

KEYWORDS: Environmental Effects on Ecosystems; Animal Behavior; Acute Toxicity Test; Lead Nitrate; Fathead Minnows.

■ Introduction

The bioaccumulation and non-biodegradable nature of heavy metals make them a major environmental concern for aquatic ecosystems. One such heavy metal is lead, which is known to be a toxic material that can disrupt biological mechanisms' ability to respond to oxidative stress. Lead exposure has also been shown to damage fish gill tissue, decreasing the amount of oxygen that can be induced into the bloodstream of fish. This causes the fish to overcompensate by increasing its heart rate and is the reason that lead pollution correlates with increased heart rates in fish.[1] High concentrations of heavy metals can also cause DNA damage in fish.[2] Although lead and other heavy metals have been shown to impact aquatic species immediately negatively, their effects are also present farther along the food chain. A study at the University of Gaziosmanpasa concluded that lead and lead poisoning could pose an additional risk to slow breeding and long-lived species, such as certain bird species, due to lead's ability to bioaccumulate in prey, such as aquatic organisms. Lead poisoning is particularly concerning for these species as it is difficult to observe because carcasses in the wild often do not last long enough for discovery, causing many poisoning cases to go unnoticed.[3]

Lead is a common pollutant produced from various sources—the largest proportion of ambient lead pollution results from mining and ore processing systems. Industrial, residential, and power generation establishments serve as a secondary but significant source of traces of lead from fossil fuel burning.[5] A study by Kontaş and Bostanci also demonstrated that mining and industry are not the only sources of heavy metals, as organic pesticides and other agricultural chemicals that run into water sources can also contain heavy metals.[2]

In a 1984 report, the EPA referenced an acute toxicity study that found LC_{50} values for lead chloride in fathead minnows (*Pimephales promelas*) as low as 5,580 µg/L.[6] The LC_{50} value describes the dose concentration of a chemical or pollutant required to fatally impact 50% of a test organism population in a given area. Based on current databases, the EPA regulates industry pollution of lead based on the maximum monthly average of lead pollutants that they have established, ranging from 0.032 to 0.41 mg/L.[7] Other environmental agencies outside of the United States also establish guidelines for lead concentrations in ambient water. However, these guidelines do not always protect aquatic life. Concentrations of heavy metals resulting from anthropogenic sources within permissible limits have been shown to cause adverse effects on the common carp (*Cyprinus carpio*) fish species.[4]

Fathead minnows are an effective test organism that has been approved by the EPA to be used for acute toxicity testing and determining guidelines for national water criteria.[15] The purpose of this study was to determine the impact of less-than-lethal doses of lead nitrate on the behavior of fathead minnows through an acute toxicity test, as well as the extent to which heavy metal pollution that is within government standards can still have notable negative impacts on the behavior of aquatic life such as limiting motor function and impairing oxidative respiration.

■ Methods

A collection of 30-40 fathead minnows was purchased from a commercial pet store and transported to the Moravian Academy Upper School Biology Lab for the experiment. All test organisms were placed in a 20-gallon holding tank equipped with a Tetra Whisper 10-30 Gallon Internal Power Filter for Aquariums and Whisper AP300 Aquarium Air Pump. Following their original placement in the holding tank, test organisms were given 14 days to acclimate to tank conditions. Following this acclimation period, test conditions were prepared, and

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testing periods consisted of 5-day acute test trials, each with 4 individual Pyrex 1L beakers. In total, five trials were completed to provide replicates for each tested concentration of lead nitrate. The testing beakers were washed with tap water and rinsed with Poland Spring Water before being filled with Poland Spring Water using a 1-liter graduated cylinder. Poland Spring Water has a pH range of 5.4-7.3, and turbidity of ND-0.16.[8] Test organisms were randomly removed from the holding tank in groups of two and placed into the beakers so that each 1L beaker filled with spring water contained two test organisms. The group of four beakers containing test organisms was moved into an isolated room where they could remain undisturbed and given a full 24-hour acclimation period before adding lead nitrate to prevent any extraneous results from general stress. Following this acclimation, lead nitrate solution (Flinn Scientific Inc.) was added to the individual beakers using a micropipette. Concentrations of 5μL/L, 15μL/L, 25μL/L, and 65μL/L of lead nitrate were added so that each container contained one of the concentrations. Treatments were assigned to test organisms at random for all trials. The test organisms were exposed to their individual concentrations of lead nitrate for 4 consecutive days. Each day, including the initial acclimation day, an iPhone SE Second Generation was used to record the beakers for a 1-minute observation period. This recording was used to gather quantitative data on fish behavior following the testing period to avoid bias and missing observations. After acquiring a recording on the 5th day of the testing period, the test organisms were returned to the holding tank using the fish net. This meant that fish used in a given trial had a chance to be randomly used again in a future trial. However, it was determined that the relatively large population of fish in the central culture, the random assignment of fish to treatment, and the acute nature of treatment periods would account for any accumulation of lead during analysis. Beakers were washed with tap water and a wire brush and placed to dry before being used again in the subsequent trial.

The parameters measured were activity duration, activity frequency, and respiration rate. For this experiment, any movement by the fish, such as swimming, rotating, or eating, was classified as activity. Activity duration was recorded as a value measured in seconds within the range of 0-60s and served as an estimation of the time one or both of the fish was actively engaged in activity based on observation. Activity frequency was measured by using observation to approximate the number of times per 1-minute observation period that either test organism engaged in activity. Respiration rate was measured by manually counting the number of times one of the test organisms engaged in respiration based upon the movement of gills and opening of the mouth. Respiration rates were often extrapolated based upon observation periods that were less than 60s as a result of test organisms not remaining in clear sight for observation for a full 60s observation period. To extrapolate a respiration rate, test organisms were observed for as long as possible, and the number of respiration breaths was measured for this smaller unit of time. The 60s observation period was divided by the time the test organisms could be observed. This value was multiplied by the number of respiration breaths taken by the test organism to determine the respiration rate per minute for the test organism.

Results and Discussion

The average values for activity duration, activity frequency, and respiration rate can be found in Table 1, Table 2, and Table 3, respectively. One listed value represents an average of all the recorded values for that specific concentration for the five days of that trial. Control trials were carried out separately from experimental trials, and only four groups were used, which caused a gap in each table. All averages were calculated and organized using Microsoft Excel.

Table 1: Average activity duration (s)

	Replicates				
Concentration (μl/L)	1	2	3	4	5
0*	45.7	46.6	44.2	47.6	
5	39	43	50	58	46
15	57	57	54	60	46
25	60	28	56	56	35
65**		30	52	48	58

Control group values are an average of two control groups from two trials carried out separately from experimental trials; values were not used twice

*Only four control groups were used

**Testing error invalidated the result of the 65μl/L group in Trial 1

Table 2: Average activity frequency (engagement per minute)

	Replicates				
Concentration (μl/L)	1	2	3	4	5
0*	11.4	10.8	7.7	9.8	
5	6.8	7	9	8.8	9.6
15	7.4	9.4	9	8.8	7.8
25	7	5	9.4	9.8	6.6
65**		5.4	7.8	6.6	8.8

Control group values are an average of two control groups from two trials carried out separately from experimental trials; values were not used twice

*Only four control groups were used

**Testing error invalidated the result of the 65μl/L group in Trial 1

Table 3: Average respiration rate (respiration per minute)

	Replicates				
Concentration (μl/L)	1	2	3	4	5
0*	105.8	142.7	116.7	108.3	
5	132.6	154.6	123	128	144
15	113.6	101.8	135.2	138	146.6
25	91.8	122	130.6	105.6	133.4
65**		123.8	122.4	127.8	135

Control group values are an average of two control groups from two trials carried out separately from experimental trials; values were not used twice

*Only four control groups were used

**Testing error invalidated the result of the 65μl/L group in Trial 1

Table 4 lists the results from the statistical analysis done to the data where n_c represents the number of observations from the control group, n_e represents the number of observations from the experimental group, μ_c represents the actual mean of the control group for that respective observational criteria, μ_e represents the actual mean of the experimental groups, t represents the t-statistic from the t-test that was carried out, and p represents the one-sided p-value that resulted from the t-test. An independent t-test was conducted for each behavioral test. Each row of listed values was calculated using the same control values as the behaviors do not impede the measurement of each other and were therefore observed from the same recordings.

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Table 4: T-test analysis results

	n_c	n_e	μ_c	μ_e	t	p
Activity Duration	32	97	45.5	49.3	-1.13	0.130
Activity Frequency	32	97	9.72	7.90	1.98	0.0267
Respiration Rate	32	97	117	127	-1.10	0.138

Results in Table 4 data values have been rounded to three significant figures for formatting purposes.

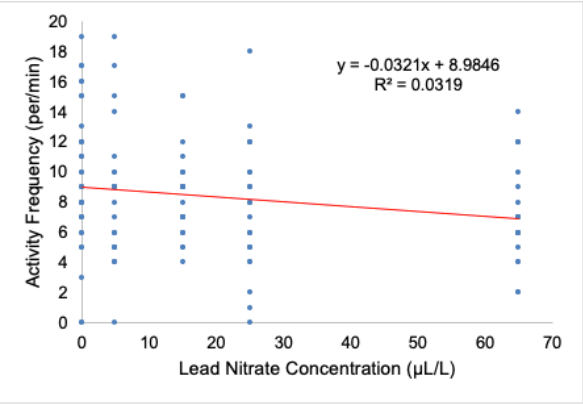

Figure 1: Least squares regression graph

The study's general findings following experimentation showed statistically significant evidence that introduction to lead nitrate inhibited the natural rate at which fathead minnows engaged in activity, thereby altering the test organism's behavioral patterns. However, while similarly predicted trends were observed in both activity duration and respiration rate data, these trends were not statistically significant.

The present study expected a decrease in activity duration in experimental groups compared to control groups, as previous studies have shown that lead exposure can cause behavioral impairment.[16] The experimental results shown in Table 1 represent the mean data points for each group and therefore do not demonstrate the trend that was noticed after experimentation. Following the implementation of t-test data analysis using Microsoft Excel, a one-tailed t-test P-value of 0.13 was derived. These results are shown in Table 4. This P-value suggested the presence of an observed trend in accordance with the original hypothesis, although it failed to do so with convincing statistical significance. While the test organisms generally tended to show decreased activity duration during acute exposure, further experimentation would need to be conducted to make statistically significant conclusions.

The present study hypothesized that activity frequency levels would decrease following the introduction of lead nitrate due to past studies that have indicated behavioral changes due to exposure to lead and other heavy metal pollutants.[1-16] Table 2 shows the means of collected data and represents the trend that aligns with the hypothesized result of exposure in that lead caused a decrease in activity frequency. Following the application of t-test data analysis on all collected activity frequency data using Microsoft Excel, a one-tailed t-test P-value of 0.027 was found, proving the data demonstrated a statistically significant trend and providing convincing evidence that lead exposure caused the observed decrease in activity frequency. These results are shown in Table 4. Regarding activity frequency, the control group averaged 9.7 engagements per one-minute observation period, while the 65 µL/L group averaged only 7.2 engagements per one-minute observation period.

Based on the statistical significance demonstrated by the activity frequency data, further statistical analysis was applied to determine the presence of a trend dependent upon concentration. A t-test for slope and regression analysis was carried out using Microsoft Excel. Figure 1 shows a scatterplot of the data for activity frequency at the 0 µL/L, 5 µL/L, 15 µL/L, 25 µL/L, and 65 µL/L concentration levels. This graph output a regression line with a slope of -0.0321, meaning that with each increase of 10 µL/L of lead nitrate, the number of engagements per minute is expected to decrease by about 0.321. The regression analysis yielded a t-statistic value of -2.05 and a P-value of 0.043 which is below the alpha level of 0.05. Therefore, these results showed convincing evidence that there was a negative linear relationship between the concentration of lead nitrate and the frequency of activity engagement observed in the fathead minnows.

The present study hypothesized that the respiration rate of test organisms would increase due to oxidative stress brought about by exposure to lead nitrate.[1-11] Table 3 shows the averages of respiration rate data collected throughout the experiment and demonstrates a trend that follows the hypothesized outcome of increased respiration rate. Following the implementation of a t-test data analysis on all collected respiration rate data using Microsoft Excel, a one-tailed P-value of 0.14 was derived. These results are shown in Table 4. This P-value demonstrated the presence of a trend, although it failed to verify it as convincing or statistically significant evidence. While the test organisms generally tended to show increased respiration rates during acute exposure, further experimentation would need to be conducted to make statistically significant conclusions.

The primary source of error present in the study was the use and re-use of fish from a central culture. While organisms were given re-acclimation time, some of the lead nitrates may have bio-accumulated within their body tissue affecting the results of later trials. However, test organisms were randomly selected from the central holding tank and assigned to treatment at random. It was determined that the acute nature of the study and the random assignment from the central holding tank allowed for this practice. Additionally, test organisms were moved to new holding containers for testing, which may have caused stress that permeated into the beginning of the trial regardless of the 24-hour acclimation period to the new holding containers.

Conclusion

While no statistically significant trends were found for decreased activity duration or increased respiration rates in experimental groups compared to control groups, the t-tests provided evidence that some trends may be present. The present study found convincing evidence that the introduction of lead nitrate caused a statistically significant decrease in activity engagement frequency over a 96-hour acute testing period,

corresponding to a negative linear relationship between lead nitrate concentration and activity frequency.

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Vincent Menichelli graduated from Moravian Academy in the class of 2022. He is 18 and will attend the Stevens Institute of Technology to study environmental engineering and pursue further research. His research interests include toxicology, renewable energy, and assessing the anthropogenic impact on environmental systems.

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