# INVESTIGATION OF THE MORPHOLOGY OF THE HEART OF ZEBRAFISH EMBRYOS EXPOSED TO DIFFERENT CONCENTRATIONS OF METHYLMERCURY

An Undergraduate Research Scholars Thesis

by

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# ABSTRACT

The Investigation of the Morphology of the Heart of Zebrafish Embryos Exposed to Different Concentrations of Methylmercury. (May 2014)

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Mercury is a well-known neurotoxicant. In its elemental form, mercury is easily distributed into the atmosphere due to its relatively low boiling point. Once elemental mercury becomes airborne it can travel long distances to eventually be deposited into soil and in all types of bodies of water, including streams, lakes, rivers and oceans where it is converted to methylmercury by bacteria. Methylmercury can reach high concentrations in predatory or long-lived fish such as swordfish and tuna, which are prime food sources for humans. Consumption of contaminated fish or marine mammals is the major route by which humans are exposed to methylmercury. We examined the effect of different levels of methylmercury exposure on heart development of wild type zebrafish embryos (ZFEs). ZFEs were exposed to one of two different concentrations of methylmercury (10 ppb (parts per billion) or 50 ppb), and to 0 ppb methylmercury, using 24-well flat-bottom plates. A minimum of 24 ZFEs were tested with each dose of methylmercury. The 24-well plates were incubated for up to 72 h at 28.5 °C. After 24 hours exposure to each concentration of methylmercury, all surviving embryos were transferred to fresh embryo medium without methylmercury (0 ppb). Images of stained sections of ZFEs exposed to three different concentrations of methylmercury (10, 50 and 0µgl) and fixed at 72hpf were captured and NIH

Image J was used for measurement and each ZFE heart was assessed for normal morphological development. No significant differences were observed between any of the groups assessed.

# ACKNOWLEDGEMENTS

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# CHAPTER I

# INTRODUCTION

Methylmercury is a form of mercury that can be harmful to the developing brains of unborn babies and young children, affecting cognitive, motor, and sensory functions. The more methylmercury that accumulates into an individual's bloodstream, the longer the exposure time, and the younger in age of the person consuming the fish, the more severe the effects may be. Elemental mercury is primarily transformed into methylmercury by sulfate-reducing bacteria that can be found in both soil and water (Guimaraes et al., 2000; Sparling, 2009). Methylmercury is the form of mercury that becomes bioconcentrated because it is better retained by organisms at all levels of the food chain due to its lipid solubility (Morel et al., 1998). Methylmercury can reach high concentrations in predatory or long-lived fish, including swordfish, tuna, king mackerel and shark, which are prime food sources for humans and other mammals, especially marine mammals (Al-Ardhi and Al-Ani, 2008; Dietz et al., 2000; Gilmour and Riedel, 2000; Mason et al., 1995; Myers et al., 2007; US EPA, 2008).

Zebrafish embryos may not only be ecotoxicologically relevant models, but may also aid in the elucidation of the molecular mechanisms underlying the effects of low-level methylmercury exposure in humans (Yang et al., 2009 and references therein). There are many advantages to using zebrafish embryos (ZFEs) in toxicity studies, including: rapid ex utero development; the chorion, which is the protective covering found over the early ZFE, is transparent; transparency of the ZFEs themselves during early embryonic development; and the ability to provide direct, accurate chemical delivery to the ZFEs at any time during development (Hill et al., 2003;

Gonzalez et al., 2005; Tanguay and Reimers, 2008). Additional beneficial factors for using ZFEs in toxicity studies include: a single pair of breeding zebrafish can generate up to 200 ZFEs in a single week; zebrafish can reach sexual maturity by approximately 3 months of age; and a great deal of information is already known concerning zebrafish genetics and developmental biology (Westerfield, 2000; Yang et al., 2007). The fact that use of ZFEs affords economic simplicity due to their small size and rapid development, the ability to easily expose developing ZFEs to different toxicants, the ability to understand both the complex genetic and developmental biology of this animal model, make ZFEs an excellent model system with which to examine the effects of possible toxicants that affect the developing cardiovascular system (Heideman et al., 2005).

The effects of methylmercury on development of the ZFE neural tube reflect the well-known neural toxicity of methylmercury, because all doses tested in this current study have been previously reported to cause decreased staining with proliferating cell nuclear antigen (PCNA), suggesting decreased cell proliferation (Hassan et al., 2012). Perry et al. (1988) reported a decrease in mitotic index and an increased percentage of abnormal mitosis in methylmercury - Treated killifish embryos. Smith et al. (2010) observed that the adult zebrafish telencephalon cell body density was significantly decreased at all developmental methylmercury exposures greater than 0.01 μM. These observations are similar to those reported by Yang et al. (2010) where exposure to methylmercury significantly impaired development of the zebrafish fin fold and the tail fin primordium. Furthermore, Yang et al. (2010) reported that concentrations of methylmercury as low as 6ppb (6 μg per liter) methylmercury and an exposure time as short as 6h caused defects in tail fin development. Cuello et al. (2012) reported that zebrafish larvae

exposed to 5 to 25 ppb methylmercury were observed to have a bent body axis and they accumulated blood in their hearts as well as presented an irregular heartbeat.

The zebrafish heart begins development in a fashion that is similar in all vertebrates two thin walled primordial cardiac tubes fuse together to form the definitive heart tube by 24 hours after fertilization and it also is around this time that contraction of the heart is initiated (Stainer et al., 1993). The fish heart has two chambers, atrium and ventricle, and blood flows first into the atrium from the sinus venosus, which receives blood from the venous end of the circulatory system . Blood then flows from the atrium into ventricle and blood is returned to bulbus arterious, which connects to the branchial arteries and the ventral aorta to return blood the rest of the circulatory system (Hu et al., 2001)

We entered this investigation with the hypothesis that early exposure to methylmercury will have an adverse effect on heart development in zebrafish embryos. We anticipate that the hearts of ZFEs that are exposed to low concentrations of methylmercury for hours 5 through 30 of post fertilization development will both show significantly reduced overall morphological growth and complexity when compared to those that have not been exposed to methylmercury during the same developmental time period.

# CHAPTER II

# METHODS

## Zebrafish Embryos

Adult wild-type zebrafish of the AB strain were raised in the Department of Biology at Texas A&M University and maintained under standard laboratory conditions at an ambient temperature of approximately 28.0 °C (based on protocols described by Westerfield, 2000). Male and female adult zebrafish were paired in the evening (4-6 p.m.) and fertilized embryos were obtained at approximately 9-10 a.m. the following morning. All ZFEs were held in an incubator with a constant temperature of 28.5 °C after transfer from the Biology Department. Embryo medium, consisting of ultrapure water containing low concentrations of specific ions and adjusted to pH 7.2, was used to maintain the developing ZFEs and was freshly prepared for each experiment according to Westerfield (2000). All ZFEs were staged and fixed at specific hours post fertilization (48 and 72 hpf) as described by Kimmel et al. (1995). Both adult and embryonic ZFEs were maintained according to protocols that were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 8523, revised 1996) and animal use was approved by the Texas A&M University Institutional Committee to Approve Animal Use Protocols.

## Methylmercury Preparation and Exposure

Methylmercuric chloride (95% purity) were obtained from Alfa Aesar (Ward Hill, MA, USA). Methylmercuric chloride (methylmercury) and initially dissolved in sterile, deionized water to a

concentration of 0.1 mg ml-1) and further diluted with embryo medium for ZFE exposure. All methylmercury stock solutions were stored at 4 °C until used. ZFEs were exposed to one of two different concentrations of methylmercury (10 ppb (parts per billion) and 50 ppb), and to 0 ppb methylmercury, using 24-well flat-bottom plates with low evaporation lids (BD Biosciences, San Jose, CA, USA). The total volume of embryo medium contained in each well of the 34-well plates, WAS 2.0 ml. In each experiment some ZFEs were placed in embryo medium without methylmercury (0 ppb) to serve as negative controls. Two or three ZFEs were added to each well and the 24-well plates were prepared in triplicate. A minimum of 24 ZFEs were tested at each dose of methylmercury. The 24-well plates were covered with low evaporation lids and incubated for up to 72 h at 28.5 °C (Thelco Laboratory Incubator; Cole-Palmer Instrument, Vernon Hills, IL, USA). After 24 hours exposure to each concentration of methylmercury, all surviving embryos were transferred to fresh embryo medium that did not contain any methylmercury (0 ppb) (Hassan, et al., 2012).

**Morphological Analysis**

Three groups of ZFEs were evaluated: Control, exposure to 10ppb and exposure to 50ppb. ZFEs from each group were anesthetized by exposure to MS-222 (a fish anesthetic) followed by rapid chilling on ice. The ZFEs were fixed in either 10% NBF or 1% Paraformaldehyde: 1% Gluteraldehyde. Once the ZFEs were fixed, 10 hatched ZFEs per group were be embedded in paraffin, sectioned sagittal at a thickness of 5 µm and stained with Hematoxylin and Eosin. The slides were coded so data collection was carried out with the investigator blinded with respect to the individual group. Heart area, pericardial area and heart wall thickness were measured. Images of stained sections of ZFEs exposed to the three different concentrations of methylmercury and

fixed at 72hpf were captured using an Eclipse E400 microscope equipped with a 40X objective, a Nikon DXM1200 digital camera and ACTI imaging software. NIH Image J was used for measurement (Abramoff et al., 2004).

**Statistical Analysis**

ANOVA was performed to assess differences among groups expressed as means±standard error of the mean (SEM). Post hoc analysis was not carried out because no data set reached statistical significance based on ANOVA analysis.

# CHAPTER III

# RESULTS

The zebrafish heart, as all fish hearts, consists of a single tube that has two major chambers, an atrium and a ventricle, which are located inside a body cavity called the pericardium (Figures 1 and 2). Blood flows from the body to the atrium, then to the thicker-walled ventricle, out through the bulbus arteriosus and ventral aorta back to the body. After 24 hours of exposure (hours 6-30 after fertilization) to one of two concentrations of methylmercury, zebrafish embryo (ZFE) hearts were examined at 72 hours after fertilization. Measurements of various parameters of the heart were taken from a minimum of seven ZFEs from each group included in this study. All scoring was done with the investigator blind to which group each embryo belonged. The volume of the heart that included the atrium and ventricle, ventricular wall thickness and pericardial cavity volume were measured.

**Heart volume**

Heart volume data are shown in Figure 3. Heart volume from control ZFEs was compared to heart volumes from ZFEs exposed for 24 hours to either 10 ppb or 50 ppb methylmercury. We expected to observe no difference when control ZFE heart volume was compared to ZFEs exposed to 10 ppb methylmercury, but we expected the hearts of ZFEs exposed to 50 ppb methylmercury to be significantly larger than control ZFE hearts. We observed no statistical difference between any of the groups analyzed, based on analysis by one-way ANOVA.

**Pericardial cavity volume**

Pericardial cavity volume data are shown in Figure 4. Pericardial cavity volume from control ZFEs was compared to pericardial cavity volumes from ZFEs exposed for 24 hours to either 10 ppb or 50 ppb methylmercury. We expected to observe no difference when control pericardial cavity volume was compared to ZFEs exposed to 10 ppb methylmercury, but we expected the pericardial cavities of ZFEs exposed to 50 ppb methylmercury to be significantly larger than the volumes observed in control ZFEs. We observed no statistical difference between any of the groups analyzed, based on analysis by one-way ANOVA.

**Ventricle thickness**

The thickness of the ventricles is shown in Figure 5. When we examined the ventricle thickness we expected to observe either no difference in the thickness of the ventricular walls between any of the groups or to observe that the thickness of the ventricle in the hearts of ZFEs exposed to 50 ppb be thinner than control ZFE heart ventricles. We observed no statistical difference between any of the groups analyzed, based on analysis by one-way ANOVA.

# CHAPTER IV

# CONCLUSIONS

In this study, we compared the morphological characteristics of the heart between three experimental groups of zebrafish embryos: Control, 24 hours of exposure to 10ppb, and 24 hours of exposure to 50ppb. While no statistically significant results were observed, several trends in these results were observed. Based on the microscopic examination of the methylmercury-exposed embryos, ZFEs exposed to the lower concentration of methylmercury (10 ppb) exhibited a strong tendency to have a smaller heart volume as well as pericardial sac volume in comparison to the controls. There was no difference in the thickness of the ventricle wall among any of the groups examined. We hypothesized that this trend towards smaller volumes could indicate a developmental delay of the heart in these ZFEs that were exposed to 10 ppb methylmercury.

It was interesting to note, that while only trends were observed, the heart and pericardial sac volumes of ZFEs exposed to the higher concentration of methylmercury (50 ppb) tended to be higher than those of ZFEs exposed to 10 ppb methylmercury. This trend could suggest that, while overall development of the heart may be delayed by exposure to methylmercury, exposure to the higher concentration of methylmercury could cause structural damage or functional deficits in the heart, which often result in cardiac edema that can cause enlargement of the heart and/or pericardial sac (Xu et al., 2002).

Although the available data are promising, they are not sufficient to draw a comprehensive picture. Future investigation may focus on the long-term effects on the structure of the heart and

provide a path for understanding the effect on the functions of the cardiovascular system such as heart rate. Disruption of heart development in zebrafish embryos exposed to methylmercury in the ppb range is of particular importance in light of the growing body of evidence demonstrating that the cardiovascular system is also a key target of methylmercury toxicity in humans. While zebrafish studies are only beginning to have an impact in the area of cardiovascular toxicology, the value of the model is unquestionable. Zebrafish research is continuing to make a substantial impact on the study of heart development.

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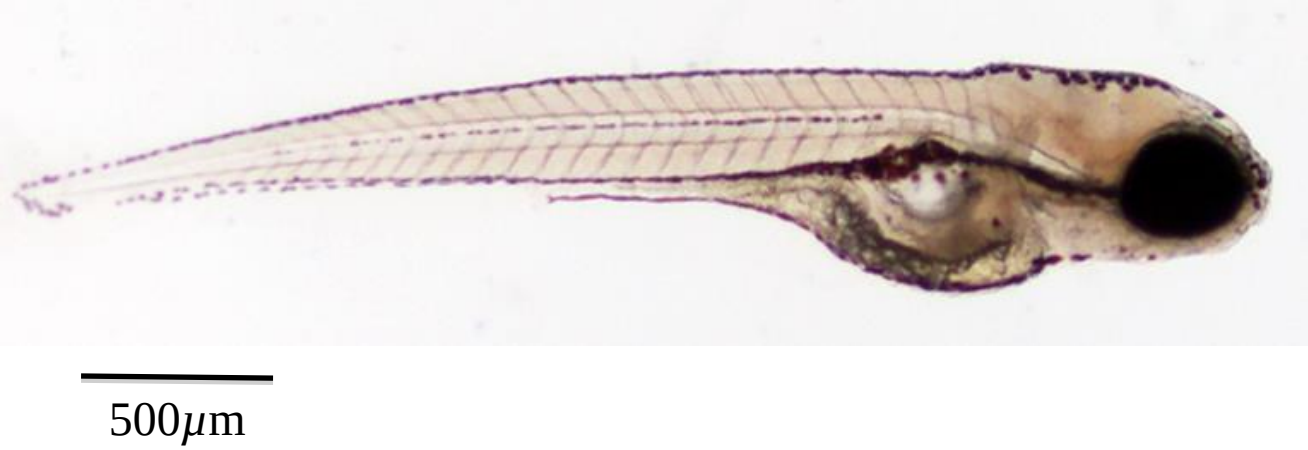


**Figure 1. Lateral view of 72 hour normal (control) zebrafish embryo.**

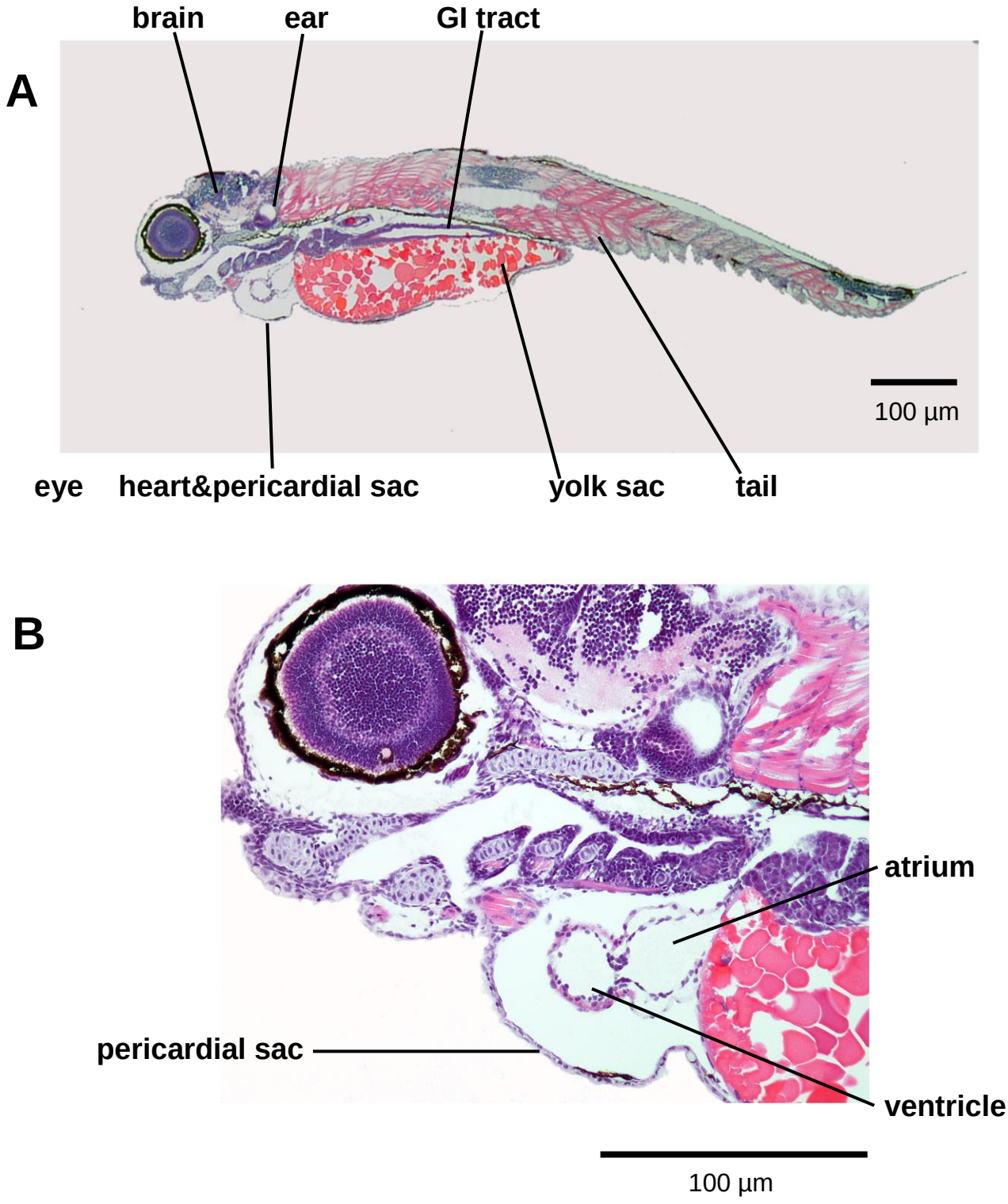


**Figure 2. Five micron thick sections of control zebrafish embryos stained with H&E. A = low magnification ; B = higher magnification of heart of zebrafish embryo seen in A.**

Volume (microns)

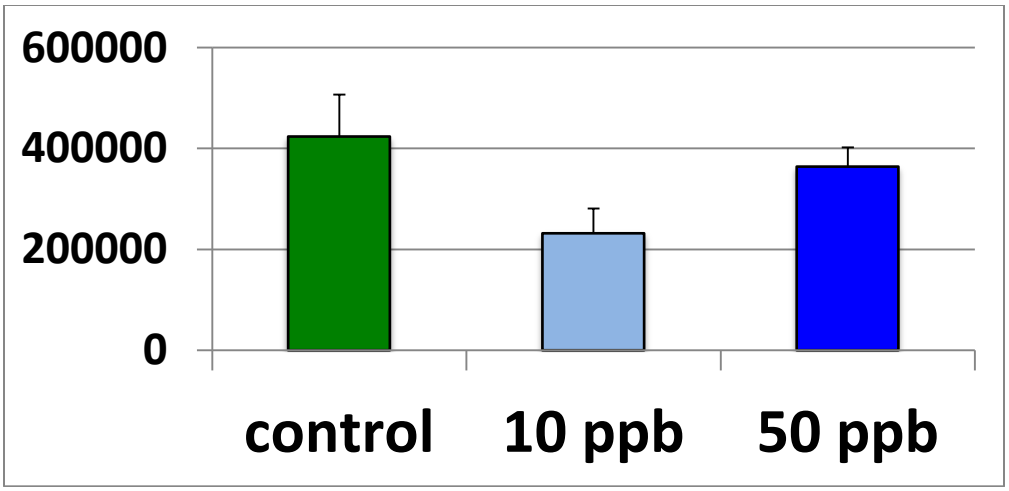


**Figure 3. Assessment of heart volume in 72 hour old zebrafish embryos. One-Way ANOVA indicates no significant differences between groups.**

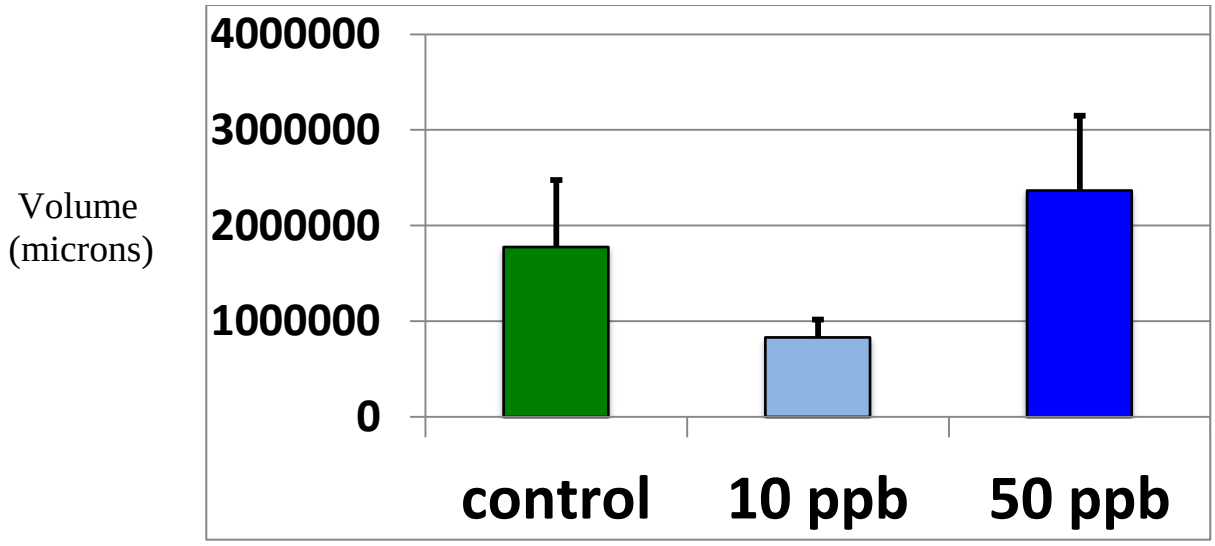


**Figure 4. Assessment of ventricular wall thickness in 72 hour old zebrafish embryos. One-Way ANOVA indicates no significant differences between groups.**

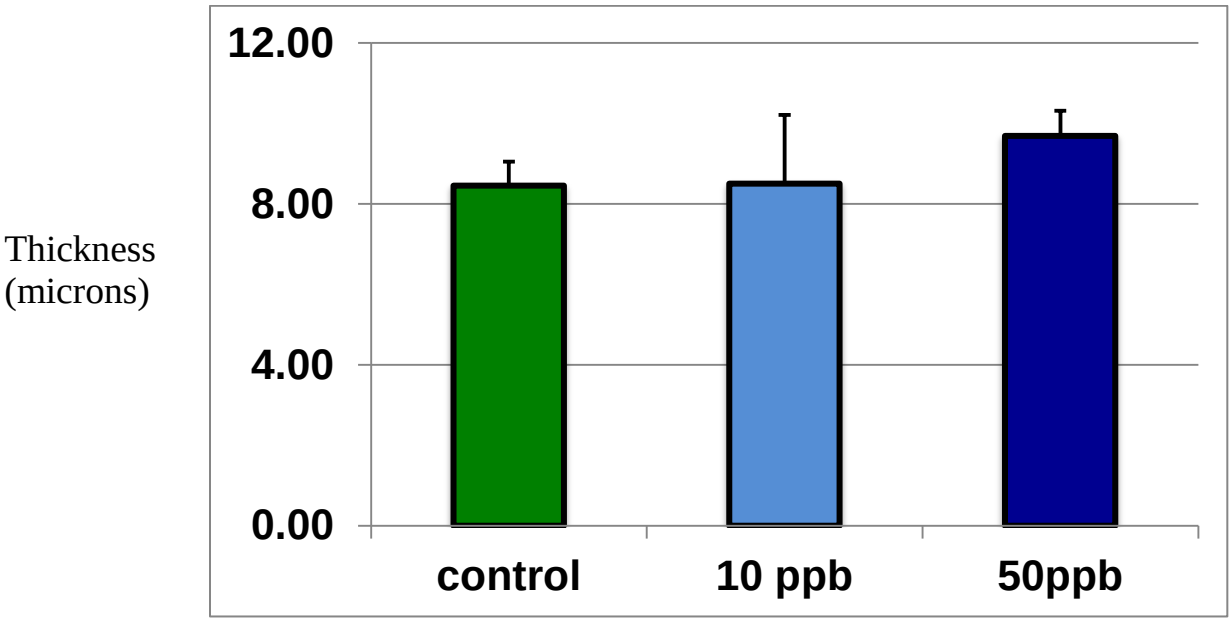


**Figure 5. Assessment of ventricular wall thickness in 72 hour old zebrafish embryos. One-Way ANOVA indicates no significant differences between groups.**