Proceedings of The National Conference
On Undergraduate Research (NCUR) 2013
University of Wisconsin La Crosse, WI
April 11 – 13, 2013

Triclosan Effects on Zebrafish Heart Rate

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Abstract

Triclosan (TCS) is a biocide, is commonly used in soaps, hand sanitizers, and detergents. Due to its extensive use, it is in now one of the top five chemicals found in surface waters. Recent research has implicated this chemical as a thyroid hormone disrupter. The goal of this research project was to determine if exposure to levels of TCS found in drinking water (20ng/L TCS) and surface water (200ng/L TCS) would impact the metabolism by altering heart rate (HR) of newly hatched zebrafish. Newly hatched zebrafish were exposed to control, 20ng/L TCS or 200ng/L TCS for 72 hours. Heart rates were counted for 30 seconds and recorded. Data was analyzed using ANOVA on the Minitab program. Our results indicate that exposure to drinking water levels (20ng/L TCS) reduced heart rate significantly, while exposure to 200ng/L TCS did not change from the control. We further explored the impact of pre-exposure to these low levels of TCS on the LC50 concentrations for the zebrafish. After 72 hours the heart rate was recorded, and the fish were taken and placed in a feeding dish (paramecium) for 2-3 hours. Fish were then exposed to various concentrations of TCS ranging from 50-400µg/L TCS for 96 hours. The LC50 for the fish in this study was significantly lower than the LC50 for adult fish found in other studies. In addition, pre-exposure to TCS did not significantly impact the effects of LC50 levels for these fish. These data indicate that further research into the effects of TCS on heart rate and metabolism should be explored.

Keywords: Triclosan, zebrafish, heart rate

1. Introduction

Triclosan (TCS) is an antimicrobial agent that targets both gram-positive and gram-negative bacteria as well as some fungi. TCS is commonly used in the following products: soaps, hand sanitizer, dish detergent, laundry detergent, toothpaste, mouthwash, deodorant, antiperspirants, cosmetics, shaving cream, face washes, shampoo, conditioners, work out apparel, surgical scrubs, plastic cutting boards, trash bags and many others (APUA, 2011). Because many of these products are topical personal care items, they wash off easily allowing the levels of TCS in surface and waste waters to increase. Since TCS use became widespread in the 1990s, it has become one of the most frequently detected compounds in waste waters. TCS has been detected in 36 U. S. streams as a result sewage efflux (Kolpin et al., 2002; EPA, 2010). The range of TCS concentrations has varied from 0.25µg/L to 6.75µg/L (Chalew and Halden, 2009). Widespread contamination is indicated by the presence of TCS in the blood of wild bottlenose dolphins (APUA, 2011).

With TCS levels in the environment so prevalent there is concern about the impact of TCS on human health and the environment. Scientists have always been concerned that TCS is leading to increased antibiotic resistance in bacteria. More recently, concern about other effects of TCS on the environment has surfaced. High concentrations of TCS have been shown to be toxic to many aquatic organisms with algae being the most sensitive (EPA, 2010). Beyond potentially eliminating the base of aquatic food chains, TCS is also toxic to larger aquatic organisms. TCS is

absorbed through the integument of aquatic organisms. It is also fat soluble and bioaccumulates in the fat stores of organisms. While environmental levels have not yet reached lethal levels for many organisms, there is no clear evidence of the impact sub-lethal levels could have on organisms. In the US, the EPA now requires that pesticide registrants add labeling statements on their produce to indicate that TCS is toxic to fish and other aquatic animals. Calafat, et al. (2008) found the median level of TCS in the urine of young girls to be 7.2µg/L. Due to concerns that TCS may affect development and reproduction and may be an endocrine disrupter the EPA is currently reevaluating TCS (EPA, 2010).

TCS has been found to decrease levels of thyroid hormones in Wistar rats and amphibians (Paul, et al., 2010; Rodreiguez, et al., 2010; Zorrilla, et al., 2009). While TCS has a structure similar to thyroxine, a hormone produced by the thyroid, it appears that reduction in thyroid hormones may be occurring as a result of liver metabolism (Paul, et al., 2010, Figure 1). Many studies have been performed looking at the effect TCS has had on thyroid-driven metamorphosis in amphibians (Hebling, et al., 2011). Research has also shown that TCS exposure can be passed to offspring in rats via breast milk (Paul, et al., 2010; Rodriguez and Sanchez, 2010).

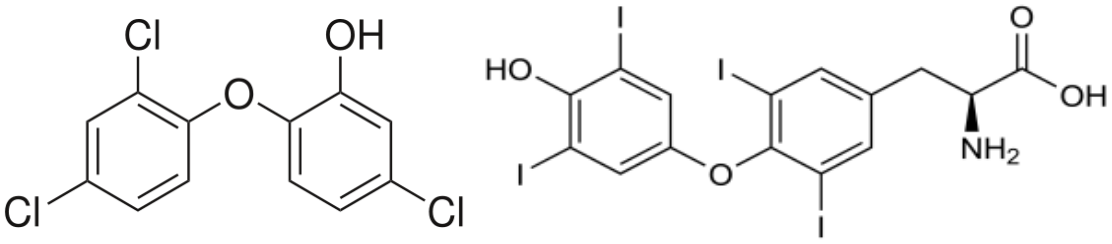

Figure 1. Structures of TCS and thyroxine.

The structure of TCS (left) and thyroxine (right).

This study was designed to measure the effect differing levels of TCS exposure has on the heart rate of recently hatched zebra fish. To simulate different environmental levels found in drinking water and surface water, TCS levels of 20ng/L (Shelver, et al., 2007) and 200ng/L (Chalew and Holden, 2009) were chosen. Zebrafish are widely used in toxicity tests and they are easy to obtain and care for in the lab setting. Also, young zebrafish are transparent, which allows for observation of their ventricle to determine heart rate (Chakraborty, et al., 2011).

2. Methods

2.1. Care Of Organisms

Zebrafish and paramecium were both ordered from Zebrafish International Resource Center (ZIRC), which is supported by grant #RR12546 from the NIH-NCRR. Approval for the care of living organisms was attained from Institutional Review Board at Central College.

Newly hatched zebrafish were used in this study. Hatching usually occurred naturally, but some fish had hardened chorions due to the solution the eggs were shipped in. Zebrafish that needed assistance hatching were squeezed gently with forceps until the chorion popped. Zebrafish were maintained at 28˚C in a solution of 0.5X E2 Embryo Medium suggested by ZIRC. Zebrafish experienced a 12D:12L photoperiod throughout the 72 hour and 96 hour exposure periods used in this study. During the 72 hour incubation the hatchlings were not fed as they were still obtaining nutrients from their yolk sac. Between the 72 hour incubation in low levels of TCS (0, 20, 200 ng/L) and the 96 hour LC50 incubation, the hatchlings were fed paramecium. Feeding beakers consisted of a 400ml beaker containing 300ml of E2 solution and 3 ml of paramecium. Paramecium cultures were kept in the lab and fed whole-wheat kernels and brewer's yeast. Fish were kept in incubators to feed for 1-3 hours before being transferred to a second exposure beaker.

2.2. Solutions

E2 is made in large quantities by mixing solutions of 100X E2A (NaCl, KCl, $MgSO_4$, KH_2PO_4, Na_2HPO_4), 500X E2B ($CaCl_2$), and 500X E2C ($NaHCO_3$). Final solution is made by mixing 100ml of 100X E2A, 20ml of 500X E2B, and 20 ml of 500X E2C a 20L liquid dispenser. Reverse osmosis water was added to reach the 19L mark, the pH was checked and adjusted to 7-7.5, and more reverse osmosis water was added to reach the 20L mark.

TCS solutions were prepared through serial dilutions to obtain the experimental exposure levels. One milligram of TCS was dissolved into one milliliter of ethyl alcohol and was transferred to a 1 liter volumetric flask. E2 solution was added to the volumetric flask to make 1 liter (1mg/L TCS). This solution was diluted using volumetric flasks and E2 solution until experimental levels of 20ng/L or 200ng/L were reached for the drinking water and surface water levels. LC50 exposure levels included 50µg/L, 100µg/L, 150 µg/L, 200 µg/L, 225 µg/L, 250 µg/L, 300 µg/L and 400 µg/L of TCS.

2.3. 72 Hour Exposure To Environmental Levels Of TCS

Thirty to forty five fish were placed into a large petri dish containing 100ml of one of E2 solution, 20ng/L TCS or 200ng/L TCS. Petri dishes were placed into incubators set at 28˚C. Each day 10ml of solution was removed and replaced with fresh solution to deter ammonia build-up. These levels were chosen because they represent levels found in drinking water (20ng/L) and surface water (200ng/L). Fish were exposed to this level for 72 hours.

2.4. Heart Rate Measurement

Following the 72 hour exposure, fish were placed into a well slide with a small amount of solution. Heart rates were visually determined using compound light microscopes and counting ventricular contraction for 30 seconds per fish. Each result was then doubled to obtain a heart rate in beats per minute (BPM).

2.5. 96 Hour TCS LC50 Determination

After heart rate determination, each fish was transferred into a feeding beaker. Fish were exposed to a second treatment of TCS for 96 hours. The original 30-45 fish in the 72 hour petri dish were distributed into three or four 400ml beakers containing a maximum of 10 fish. To determine LC50 concentrations, these fish were then exposed to TCS for 96 hours at 28˚C. This allowed for comparison of the effects of pre-exposure to control, 20ng/L and 200ng/L on the LC50. After 48 hours of exposure, fish were checked and any dead zebrafish were removed. After 96 hour exposure, the heart rates of the surviving fish were determined using the same method as the pre-exposure treatment. These experiments were repeated at least three times.

2.6. Statistical Analysis

An ANOVA was used to determine significant differences between heart rates of the zebra fish exposed to TCS compared to control. Following ANOVA, Tukey and Fisher statistical grouping methods were utilized to illustrate significance. A PROBIT model was used to determine the LC50 values for 96 hour exposure. Because sample size varied from trial to trial we were outside the heterogeneity values of this model.

3. Results

Exposure to TCS for 72 hours at 20ng/L significantly ($p = 0.000$) decreased heart rate compared to control. Exposure to 200ng/L had no significant effect on heart rate relative to the control, though it was significantly higher than the 20ng/L group (Figure 2.)

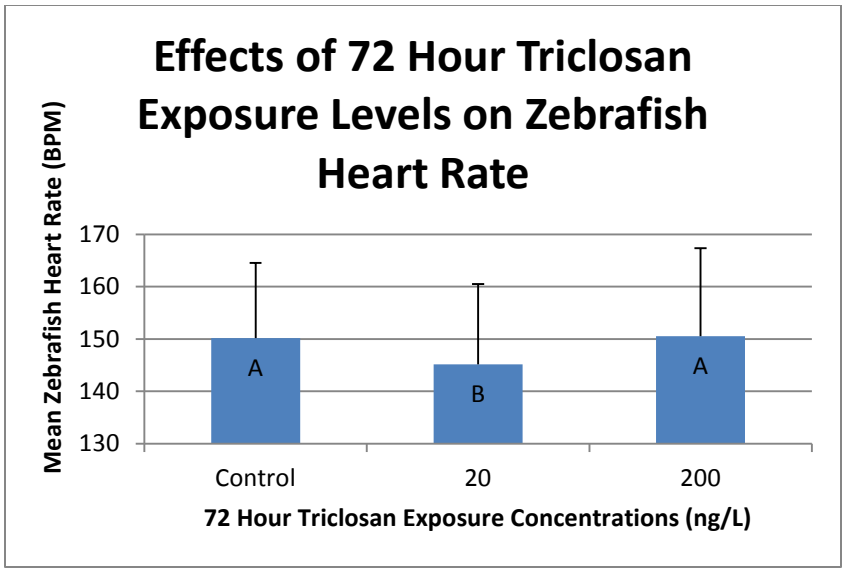

Figure 2. Effects of 72 hour TCS exposure levels on zebrafish heart rate.

Those groups having different letters are significantly different from each other at $p<0.05$.

Exposure to higher levels of TCS was performed to determine the 96 hour LC50. Test with various levels of TCS produced a trend line with heart rate decreasing as the TCS exposure level increased. ANOVA results indicate that the difference is significant for all LC50 exposure concentrations ($p= 0.000$) regardless of the 72 hour TCS exposure (Figure 3). Control and 50µL TCS exposure were found to be statistically similar (group A) and statistically different from 100µg/L and 150µg/L (group B). The 200µg/L TCS exposure results (group C) were significantly different from both groups A and B. Because only 2 fish survived the 250µg/L level, the sample size was too small for statistical analysis and is grouped as ABC.

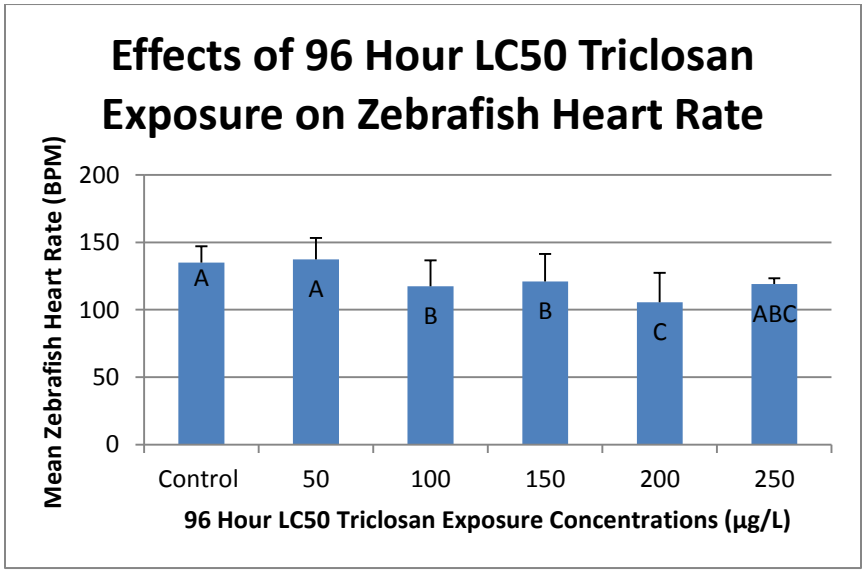

Figure 3. Effects of 96 hour LC50 TCS exposure on zebrafish heart rate.

Those groups having different letters are significantly different from each other at $p<0.05$. The reported heart rates for each LC50 group are the average of all surviving fish regardless of previous exposure levels.

The results of exposure to environmental levels of TCS (72 hour exposure) prior to LC50 exposure significantly altered heart rate in the 50µg/L LC50 exposure ($p = 0.009$). In the 50µg/L 96 hour LC50 test, fish previously

exposed to 20ng/L for 72 hours had significantly decreased heart rate compared to the control. This follows the same trend as the results from the 72 hour exposure (Figure 4). However there was no significant statistical difference in heart rate between 72 hour exposure groups in every other LC50 group (p= 0.404 for control, p=0.756 for 100µg/L, p=0.347 for 150µg/L, p=0.429 for 200µg/L). Figure 5 shows the lack of this trend in the levels directly above (150µg/L) and below (200µg/L) our 96 hour LC50 value of 192µg/L.

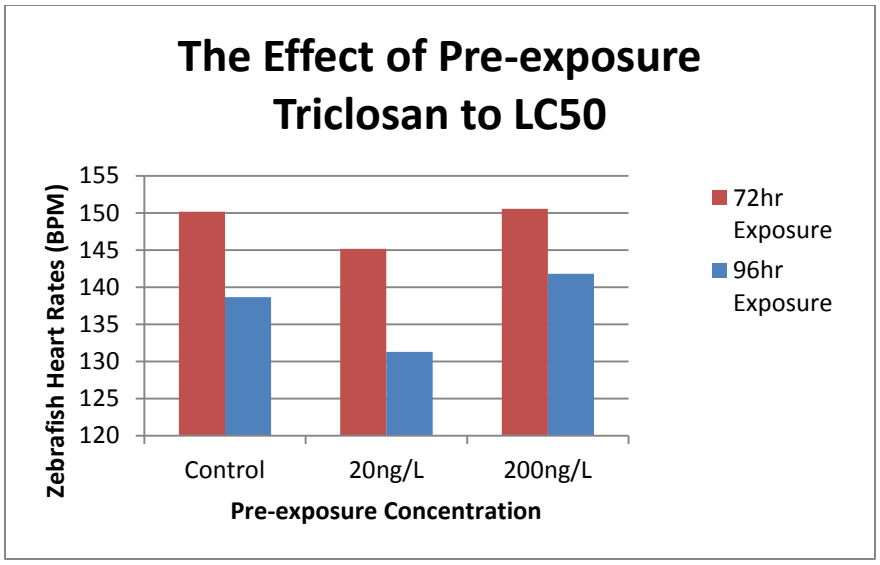

Figure 4. The effect of TCS pre-exposure on 50µg/L TCS 96 hour LC50 heart rates.

Each exposure was tested for significance using different ANOVA tests. The same trend can be seen between the two different exposures.

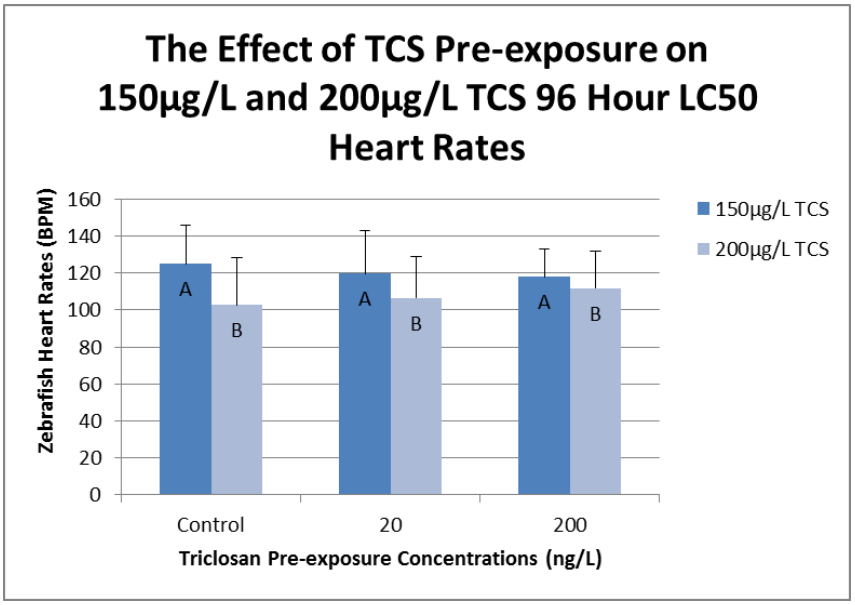

Figure 5. The effect of TCS pre-exposure on 150 and 200ug/L TCS 96 hour heart rates.

There was no significant difference between pre-exposure levels within each LC50 level as shown by letters that show significance groupings.

An LC50 value was calculated comparing survivorship at each exposure level to control data. Using a PROBIT model, we obtained an LC50 of 192µg/L with a range of 145-214µg/L at the 95% confidence level. There was no significant statistical difference in survivorship between pre-exposure groups in each LC50 group ($p= 0.728$ for control, $p=0.251$ for 50µg/L, $p=0.650$ for 100µg/L, $p=0.234$ for 150µg/L, $p=0.974$ for 200µg/L).

4. Discussion

TCS exposure at 20ng/L significantly decreased heart rate in zebrafish hatchlings. This is a level of TCS that was found in drinking water (Shelver, et al., 2007). TCS has been linked to endocrine disruption and a decrease in thyroid hormone levels (Paul, et al., 2010; Rodriguez, et al., 2010; Zorrilla et 2009). Roef et al. (2013) demonstrated a strong positive correlation between levels of thyroid hormone and heart rate and cardiac muscle contraction in men and women. We do not currently measure TCS levels in drinking water in the United States, but we do see significant levels of TCS in surface and waste waters.

The increase in heart rate relative to the control group heart rates during exposure to 200ng/L TCS tests may be a result of a stress reaction from a 10 fold higher level of TCS. During stress reactions the body releases catecholamines which increase heart rate. These results are comparable to results found in other studies on zebrafish and amphibian larvae (Oliveira, et al., 2009; Palenske, et al., 2010).

During the LC50 exposures, heart rate decreased as TCS levels increased. This drop in heart rate is most likely due to the physiological changes that occur under extreme stress as routinely leads to death in LC50 studies. In addition to decreased heart rate, we observed sluggishness and deformities in the fish. This increase in deformity rate was also observed by Oliveira, et al. (2009).

To our knowledge this is the first study to conduct LC50 96 hour tests on fish that were previously exposed to TCS at drinking and surface water levels. The 50 µg/L LC50 exposure appeared to be low enough that it did not alter the effects seen during the 72 hour exposure tests. However each concentration of LC50 exposure above the 50µg/L appears to contribute enough stress to obfuscate the effect of low levels (20ng/L) of TCS exposure for 72 hours.

In the study by Oliveira, et al. (2009) 96 hour TCS LC50 values were calculated to be 420µg/L for zebrafish embryos and to be 340µg/L for zebrafish adults. The higher LC50 value for embryos may be due to the protection provided by the egg. Chalew and Halden (2009) reported an LC50 range of 260-440µg/L for a variety of fish. In this study, hatchlings were exposed to TCS and the LC50 was calculated to be 192µg/L. The younger age of the zebrafish used in this study could make them more susceptible to smaller exposure levels and could contribute to this lower LC50 value. This is consistent with studies found in younger life stages of amphibians (Palenske, et al., 2010).

The results of this study support the research that indicates that TCS may reduce heart rate through its effect on reducing thyroid hormone levels. While the drinking water and environmental levels of TCS have not been shown to have an effect on human health, it is worth noting that TCS does bioaccumulate and exposure of humans to TCS has been increasing in recent years. If studies on TCS using zebrafish and Wistar rats represent the effects seen in humans, we should be concerned about the effects of environmental levels of TCS on human health, especially in children.

5. Conclusion

Our study indicates that exposure to low levels of TCS (20ng/L) similar to what has been detected in drinking water decreases heart rate in zebrafish hatchlings. We further demonstrated that the LC50 for hatchlings is much lower than the reported values for zebrafish embryos and adults. This indicates that the young are more susceptible to the negative effects of TCS at lower levels. Considering the levels of TCS found in the urine of young girls and the effect it has on hormone disruption there is a need to expand research into the effects of TCS. Future studies are needed to fully understand the effects of TCS on young zebrafish. We hope to conduct further research that looks at the variance in heart rates among hatchlings exposed to levels of TCS ranging from 0-500ng/L. This might ascertain if there is a dose-response trend between heart rate and TCS levels. It may be possible to determine which level of TCS exposure reverses the decrease in heart rate. In addition, further studies could assess the effect of TCS on deformities. It is clear that TCS exposure does affect aquatic biota and deserves further investigation.

6. Acknowledgements

Funding for this study was provided by the Monticello Fund. The authors would also like to thank Dr. Ellen Du Pre and Dr. Nicole Palenske for their help with research and editing.

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