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ASSESSING THE GENOTOXICITY OF TRICLOSAN IN TADPOLES OF THE AMERICAN
BULLFROG, *LITHOBATES CATESBEIANUS*.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Biology
at Virginia Commonwealth University.

by

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May 2012

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Table of Contents

	Page
Acknowledgements	iii
List of Figures	vi
Abstract	1
Chapter	
1 Introduction and Review of the Literature	2
1.1. Global Amphibian Declines	2
1.2. Genotoxicity	3
1.3. Micronucleus Bioassay	3
1.4. Triclosan	4
1.5. The American Bullfrog (<i>Lithobates catesbeianus</i>).....	11
1.6. Endpoints and Concentrations	13
2 Materials and Methods	14
2.1. Chemicals	14
2.2. Animals.....	14
2.3. Tadpole Care.....	15
2.4. Preparation of Chemical Concentrations.....	15
2.5. Blood Smear Preparation and Analysis	16
2.6. Statistical Analyses	18
3 Results	19
3.1. Micronucleus Induction Counts.....	19

3.2. Statistical Analysis by Treatment	21
3.3. Micronucleus Induction via Dose-Dependence.....	24
3.4. Length Effects of Treatments	25
4 Discussion	30
4.1. The Genotoxicity of Triclosan to <i>L. catesbeianus</i>	30
4.2. Modeling Genotoxicity.....	31
4.3. Size Effects	33
4.4. The Need for Regulation	34
4.5. Future Directions	36
Literature Cited	38
Appendix A – R scripts and outputs.....	43

List of Figures	Page
Figure 1:	6
Figure 2:	7
Figure 3:	12
Figure 4:	17
Figure 5:	22
Figure 6:	23
Figure 7:	26
Figure 8:	27
Figure 9:	28
Figure 10:	29

List of Tables	Page
Table 1:	20

List of Abbreviations

AIC	Akaike Information Criterion
ANOVA	Analysis of Variance
CYP	Cyclophosphamide
EC50	Half Maximal Effective Concentration
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
GREAT-ER	Geography-referenced Regional Exposure Assessment Tool for European Rivers
LC50	Lethal Concentration 50
LOEC	Lowest Observed Effect Concentration
MN	Micronucleus
NIH	National Institute of Health
NOEC	No Effects Concentration
PEC	Predicted Effect Concentration
PNEC	Predicted No Effect Concentration
SSD	Species Sensitivity Distribution
SVL	Snout-to-Vent Length
TCS	Triclosan
TL	Total Length

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Abstract

Amphibians are particularly sensitive to environmental degradation and, therefore, serve as effective environmental quality indicators. Research has suggested that amphibian declines are exacerbated by manmade environmental toxicants, especially those found in high concentrations in urban areas. The NIH has pinpointed genotoxicity as a major route of cancer causation, and has since developed stringent testing procedures for potentially hazardous chemicals. One such method, recognized for its simplicity and economy, is the micronucleus assay. A study was conducted assessing the genotoxicity of the widely used antimicrobial agent Triclosan to American Bullfrog tadpoles. *Lithobates catesbeianus* tadpoles were reared in glass aquaria containing ultra-high purity water and were dosed with nominal concentrations of 2.3 µg/L, 23 µg/L, and 230 µg/L Triclosan, reflecting 1x, 10x, and 100x concentrations of the compound as found in US surface waters. Eight replicates of each of the three levels of Triclosan contamination were prepared, as well as eight replicates per control group. Each replicate contained three tadpoles in a glass aquarium, from which one tadpole per tank was sampled after 1, 8, or 15 days following initial exposure to test compounds. Erythrocytes were prepared on slides and scored for micronucleus presence under 1000x magnification. Triclosan induced significant micronucleus formation after only 24 hours in all treatments relative to the negative control and exhibited a maximum of 15 micronuclei per 2,000 erythrocytes scored. Modeling of MN induction dynamics by treatment suggested that the best predictor of micronucleus induction was the acute TCS exposure level, as described by a linear mixed effects model including a binomial term of time exposed. Micronucleus induction was TCS concentration dose-dependent. This study supports that Triclosan induces significant genetic damage at environmentally relevant concentrations. It is clear that the effects of genotoxic agents must be certified so proper regulatory protocols can be developed and enforced, in order to conserve wildlife and promote human health.

CHAPTER ONE

Introduction and Review of the Literature

1.1 - Global Amphibian Declines

Amphibians are sensitive to environmental degradation because they breathe through their skin, oviposit in water, and persist in aquatic habitats at least until metamorphosis. This life history subjects the developing embryos to the quality, or lack thereof, of the pools, ponds, and streams they inhabit. Of the 5,743 described species of amphibians, 1,856 (32.5%) are listed as globally threatened (IUCN, 2004). These declines are happening quickly, and many “enigmatic-decline” species have been identified, having no known cause of decline. Many studies cite the fungal skin disease chytridiomycosis, caused by the fungus *Batrachochytridium dendrobatidis*, as responsible for some of these otherwise unexplainable declines (e.g. Berger et al., 1998; Lips, 1999; Daszak et al., 2003) and others attribute declines to increasing severity in environmental conditions due to climate change (e.g. Pounds, 1999). Habitat loss/fragmentation (Weygoldt, 1989) and over-exploitation of amphibians for human consumption have also been cited as contributing factors (Houlahan et al., 2000). It is likely that declines are occurring as a combined effect of anthropogenic and environmental stressors.

Other research has suggested that man-made environmental toxicants may be directly contributing to amphibian declines (Carey and Bryant, 1995). Amphibians are extremely sensitive to several widely-used chemicals that are associated with areas of high human population density, such as pyrethroids (insecticides), phthalates (plastics), and

Triclosan, the latter two of which are known to cause endocrine disruption in frogs leading to hermaphroditism (Oehlmann, 2009), changes in rate of metamorphosis (Veldhoen, 2006) and, in some cases, behavioral symptoms that would decrease survival rates (Palenske et al., 2010).

1.2 - Genotoxicity

A genotoxin is a chemical or compound that causes deleterious effects to the DNA of an organism. Genotoxicity was first linked to carcinogenic agents in the early 1970's as the emergence of new cancers was attributed to chemical and environmental contaminants and ultraviolet light irradiation. Research by the NIH assessing the genetic toxicity of numerous environmental agents has since pinpointed genotoxicity as a major route of cancer causation, and has led to the development of stringent testing procedures for potentially hazardous chemicals. There is a wide array of possible tests for the genotoxicity of a compound, making necessary the identification of appropriate testing procedures based upon properties of the chemical being tested. One such method, recognized for its simplicity and economy, is the micronucleus assay.

1.3 - The Micronucleus Bioassay

The micronucleus test is a visual assessment of the genotoxicity of a compound to an organism. Micronucleus (MN) formation can occur in the dividing cells of any species (Heddle et al., 1991) and is caused by structural or numerical chromosomal abnormalities (Heddle, 1973). Studies by Hayashi et al. (1998) evaluated the use of aquatic organisms to

assess the genotoxicity of water in both laboratory and field studies and found them to be viable indicators. The MN assay is an efficient screening system for clastogenic (chromosome breakage and formation of acentric fragments) and aneugenic (chromosome lagging and alterations of spindle) effects (Heddle et al., 1983) (Figure 1) and can be applied to cells *in vivo* or *in vitro*. It is widely recognized that aneuploidy is an integral component in the development of human tumors and the acquisition of malignancy (Duesberg and Rasnick, 2000). When aneuploidy or clastogenesis occurs in a cell via induction by a genotoxic agent, a MN is left as an aggregation of lagging chromosomes or chromosomal fragments, respectively, and presents as chromatin-containing structure juxtaposed to the parental nucleus. The assay has been successfully used in many amphibian species (e.g. Fernandez et al., 1993) and fish species (e.g. Oliveira et al., 2009) to detect the presence of mutagens in fresh water. In a field study by Saleh and Zeytinoglu (2001) the MN assay was shown to be effective for detecting the genotoxicity of chemical agents in frogs, and other studies have found that MN counts in tadpoles are increased following exposure to copper and lead, pyrethroids, and phthalates (Kryukov, 2000; Campana et al., 2003; Liney et al., 2006).

1.4 - Triclosan

Triclosan (hereafter TCS) is a chlorinated biphenyl ether (Figure 2) and was patented in 1964 as an antibacterial and antifungal agent which targets the enoyl-acyl carrier protein reductase (*fabI*) component of the type II fatty acid synthase system. Through up-regulation of *fabI* expression or spontaneous missense mutation of the *fabI*

gene, TCS resistant bacterial mutants can arise, which contradicts the preliminary assessment of TCS as a direct membrane disruptor and further suggests that its widespread use will select for resistant bacterial populations (Heath et al., 1998). Despite implications of its overuse, TCS has been added to many consumer products to prevent bacterial contamination; it is now included in three-quarters of all liquid hand soaps, and is added to toothpastes, plastics, children's toys, and even clothing. Its widespread use and distribution has led to increasingly large concentrations of the substance in runoff and household wastewaters.

Of the 95 chemical contaminants surveyed in a 1999-2000 study, Triclosan was one of the top 5 most frequently detected compounds in US streams (Kolpin et al., 2002). Though wastewater treatment removes some TCS from water leaving treatment facilities, exported concentrations are able to produce noticeable damage to wildlife. While a 2002 report on TCS and its use in consumer products by the European Commission's Scientific Steering Committee concluded that TCS absorbed by the human body is rapidly excreted and no long-term accumulation occurs (EC, 2002), TCS is a lipophilic compound and has been detected in human breast milk (Adolfsson-Erici et al., 2002), clearly suggesting long-term bioaccumulation potential. In addition, TCS bioconcentrates in other non-target organisms such as fish species in Sweden (Adolfsson-Erici et al., 2002) and in *Cladophora* spp. algae where it has been shown to influence both the structure and function of algal communities downstream from treated wastewater efflux (Wilson et al., 2003). It has been suggested that some of the TCS leaving wastewater facilities is a methylated form, methyl-TCS (Lindstrom et al., 2002) which can be converted by photolytic degradation to a

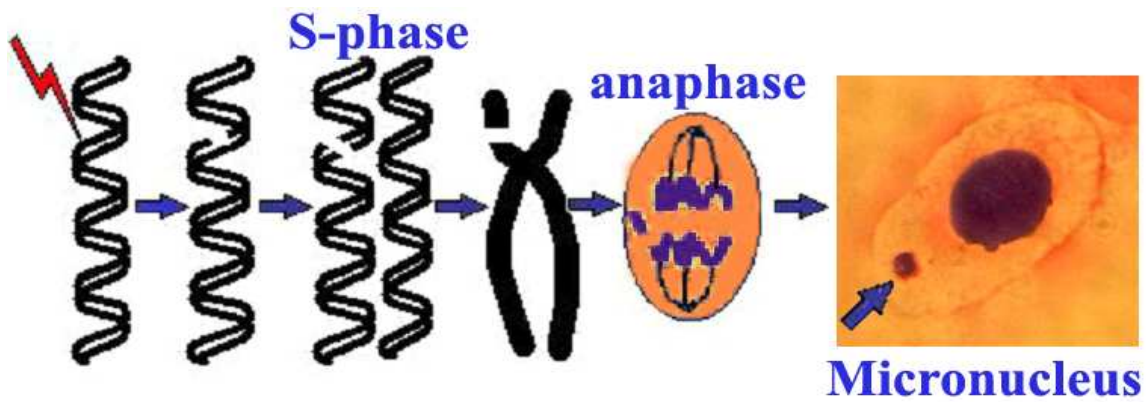


Figure 1 – Induction of a micronucleus. Mutagenesis induces clastogenesis (depicted) or aneuploidy causing a chromatin-containing fragment or a lagging chromosome to remain in the cell as a micronucleus, juxtaposed to the parental nucleus.

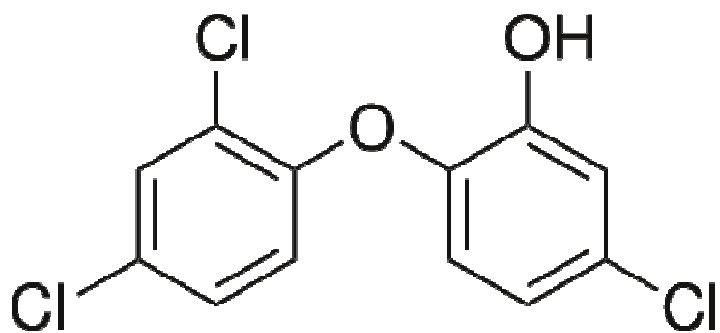


Figure 2 – Chemical Structure of the antimicrobial Triclosan ($C_{12}H_7Cl_3O_2$)

“super-chlorinated” dioxin congener (2,8-dichlorodibenzo-p-dioxin, DCDD) under laboratory conditions (Aranami and Readman, 2007). Dioxins are known carcinogens and genotoxins for a wide array of animals including humans. The contribution of TCS-derived dioxins to the total pool of environmental dioxins has increased to as much as 31% by mass in recent years (Buth et al., 2010).

Additionally, studies have demonstrated that TCS may influence the structure and function of algal communities in streams receiving treated wastewater effluent (Wilson et al., 2003) and trophically amplified “bottom-up” effects of antibacterial residues on microbial processes can alter whole ecosystems (Yang et al., 2008). In 2008, Yang et al. compared the growth-inhibiting effects of 12 different antimicrobial compounds and found that TCS was the most toxic compound in the study (NOEC [lowest concentration to show no observed effect] = 200 ng/L) for *Pseudokirchneriella subcapitata*, a freshwater microalga. Concentrations greater than 200 ng/L have been found in rivers in Germany, England, and Switzerland (Singer et al., 2002; Lindstrom et al., 2002; Sabaliunas et al., 2003; Wind et al., 2004) and TCS concentrations in US wastewater effluent were found to range from 200 ng/L to 2700 ng/L (Reiss et al., 2002). Similar concentrations were found by the U.S. Geological Survey when 139 streams across 30 states were tested between 1999 and 2000, and TCS was found to be present in 85 of the streams at a maximum concentration of 2.3µg/L (Kolpin et al., 2002). Using a Geography-referenced Regional Exposure Assessment Tool for European Rivers (GREATER), Capdevielle et al. (2007) estimated a PEC (Predicted Environmental Concentration) for the UK of 200 ng/L and through the Pharmaceutical Assessment and Transport Evaluation model (PhATE) found

that mean concentrations in North America may be as high as 850 ng/L. Capdevielle et al. (2007) created a species sensitivity distribution (SSD) from a meta-analysis of toxicity data on 13 aquatic species; three fish, four invertebrates, five algae, and a macrophyte; and derived a chronic PNEC (Predicted No Effect Concentration) of 1550 ng/L for TCS for the aforementioned organisms. While algae are particularly sensitive to acute direct effects of TCS, the potential for accumulation in fish and amphibians exceeds that of algae and invertebrates because of greater organism lipid content and lower chemical depuration rates (LeBlanc, 1995), supporting the notion that sublethal effects of TCS are of particular concern in higher organisms.

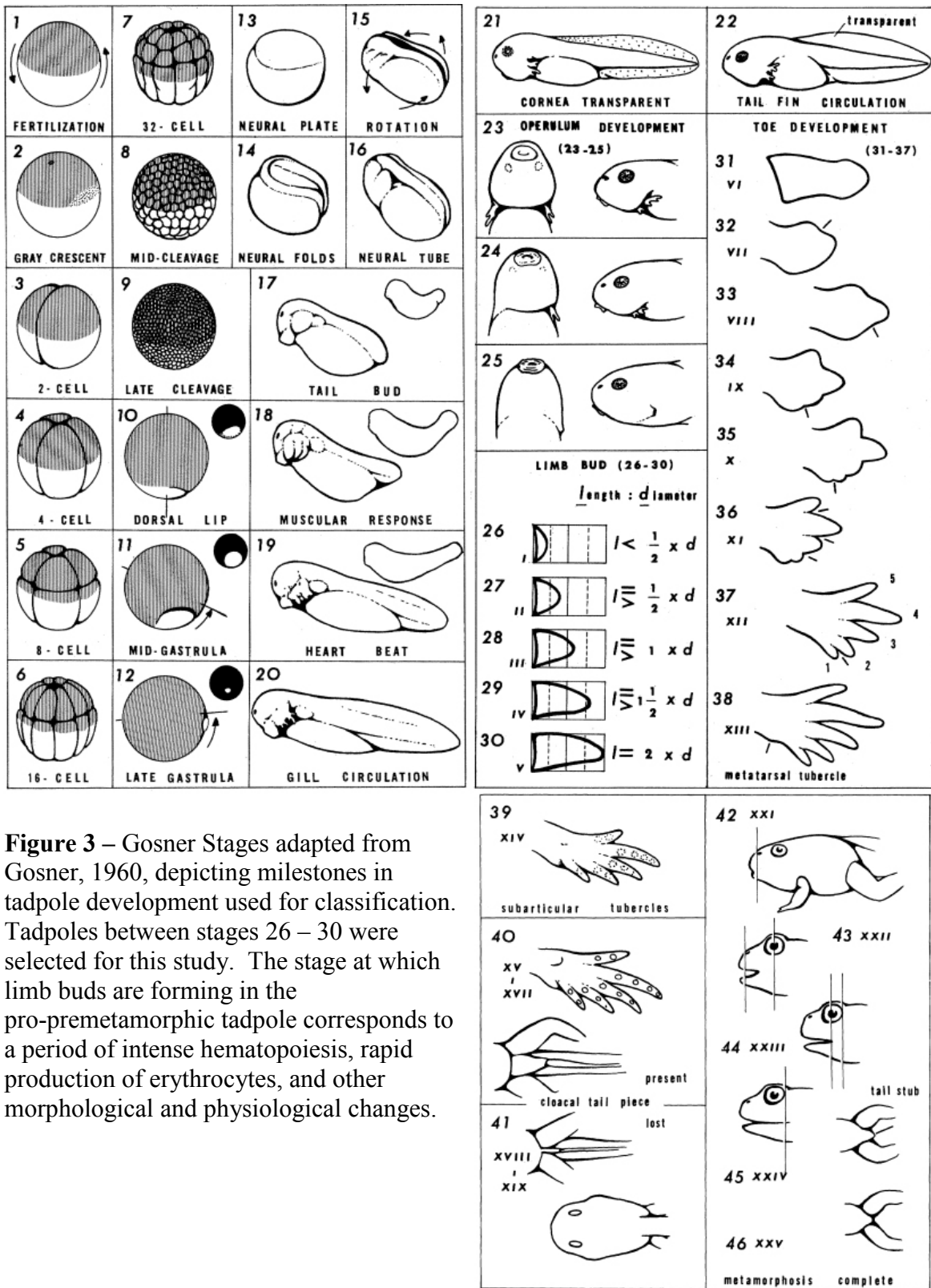
In addition to the potential for bioaccumulation and sublethal effects of TCS on wildlife and humans, direct lethal effects of TCS have also been established. TCS showed 96h LC50 (concentration required to kill 50% of exposed animals in given period of time) value of 340µg/L to adult *Danio rario* Zebrafish and a 96h LC50 value of 420µg/L for Zebrafish embryo/larvae while inducing delayed embryonic otolith formation, delayed onset of eye/body pigmentation, spine malformations, and pericardial oedema in treatment survivors (Oliveira et al., 2009). Another study found EC50 (concentration shown to have an effect on 50% of exposed animals) or LC50 concentrations between 240 and 410 µg/L for Fathead minnows (*Pimephales promelas*), bluegill sunfish (*Lepomis macrochirus*), and the water fleas *Daphnia magna* and *Ceriodaphnia dubia*, and similarly found that TCS-exposed fish also showed sublethal symptoms of loss of equilibrium, locking of the jaw, quiescence, and erratic swimming movements (Orvos et al., 2002). The behavioral changes induced in this study support narcosis as the mechanism of action of TCS and

further analysis also suggested that an interaction between environmental pH and TCS toxicity may exist, such that the neutral TCS species prevailing at a pH of 7 is more toxic than the ionized species at pH 8.5 when *Ceriodaphnia* were exposed to the treatments. Since molecules are believed to be less likely to cross lipid membranes when in their ionized state (Lipnick, 1995), and since the physiochemical properties of TCS demonstrate that it is a highly lipid-soluble compound which readily ionizes at environmental pH, this may suggest direct toxicity of TCS is maximized when pH is <8.5 and TCS is predominantly in its neutral form (Orvos et al., 2002). In a study published in 2005, a concentration of 2.3 µg/L TCS caused significant mortality of *Bufo americanus* tadpoles over a 14 day exposure period (Smith and Burgett, 2005). It is important to note, for comparison, that the concentration of TCS found in soaps is 0.2-0.3% (2g/L=2,000,000 µg/L) (Nussbaum, 2008) and that the concentration used in hospital applications is ten times higher (Perencevich et al., 2001). Humans are exposed to far higher concentrations of TCS than the animals in these studies.

The use of TCS is currently under review (again) by the FDA, which has suggested previously that it is not genotoxic to humans. Triclosan is not currently regulated by the EPA. While the genotoxicity of TCS is still under debate, it is evident that more studies need to be conducted assessing the impacts of TCS and its byproducts in natural systems and in animal models. Since the effects of TCS seem to vary between organisms, utilization of widely distributed aquatic vertebrate animal models may be useful to proxy effects and effective ambient concentrations of TCS under environmental conditions.

1.5 - The American Bullfrog, *Lithobates catesbeianus*

Lithobates catesbeianus (formerly *Rana catesbeiana*) was selected for use because its large size allows for adequate erythrocyte sampling, because it is a widely distributed species (Goin et al., 1978), and because considerable data are available about its biology. Native to North America, this frog has been introduced, intentionally and unintentionally, to over 40 countries worldwide; spread over four continents. While Bullfrogs occupy a wide range of aquatic habitats including lakes, ponds, swamps, bogs, backwaters, reservoirs, marshes, brackish ponds and ditches; research has suggested that they may have a preference for highly artificial and highly modified habitats, especially those that exhibit human-driven habitat modification (Wright and Wright, 1949; Doubledee et al., 2003). Bullfrog tadpoles have a larval period that varies from a few months to three years (Bury and Whelan 1984); larval period being negatively correlated with mean length of the frost-free season (Casper and Hendricks, 2005). Tadpoles at Gosner stage 26-30 (Figure 3) experience rapid larval growth and development of the hind limbs during a period of intense hematopoiesis and physiological change (Gosner, 1960). Previous work with *Lithobates catesbeianus* has found a maximum induction of micronuclei in circulating erythrocytes via ultraviolet irradiation at about two weeks after initial exposure (Krauter et al., 1987). A study assessing the genotoxicity of the fungicide Artea 330EC to *Rana saharica* larvae found a similar maximum in MN frequency at 12 days following exposure (Bouhafs et al., 2009).



1.6 - Endpoints and Concentrations

This laboratory investigation was conducted to determine the extent to which ecologically relevant concentrations of TCS directly induce genotoxic damage to frogs as measured by the MN assay. With the aforementioned studies in mind, and with the support of the studies which found similar or earlier maxima for MN induction in a variety of aquatic animal models following exposure to potentially genotoxic compounds (e.g. Saleh and Zeytinoglu, 2001; Kryukov, 2000; Campana et al., 2003; Oliveira et al., 2009; Jiraungkoorskul et al., 2007), it was determined that three endpoints would be established for this study; at 1 day, 8 days, and 15 days following initial exposure to TCS. The concentrations of TCS used were established to mimic current ecologically relevant concentrations as found through survey data. A concentration of 2.3 μ g/L was selected as the lowest concentration of TCS used in my study and two incrementally ten-fold higher concentrations were selected as ‘worst case scenarios’ to assess the impact that increased concentrations of TCS may have in frog larvae. Tadpoles were also treated with Cyclophosphamide (hereafter CPY), a known genotoxin, as a positive control for detecting the presence of micronuclei (Simula and Priestly, 1992; Campana et al., 2003). It was predicted that there would be acute genotoxic effects of TCS at all levels of TCS contamination, and that this effect, as measured by the MN assay, would be dose-dependent.

CHAPTER TWO

Materials and Methods

2.1 - Chemicals

Triclosan (IAPUC name: 5-chloro-2-(2,4-dichlorophenoxy)phenol), CAS No. 3380-34-5 (Figure 2), was obtained from KIC Chemicals (Armonk, NY, USA). Cyclophosphamide (CP - Genuxal[®], CAS No 50-18-0), was obtained from ASTA Medica (AG, Frankfurt, Germany) and was used as a positive control at a concentration of 5 ppm (mg/L). All test solutions were prepared immediately before experimentation.

2.2 - Animals

Lithobates catesbeianus larvae were provided by Carolina Biological Supply Company (Burlington, North Carolina, USA) and were acclimatized for 15 days prior to the start of experimentation. The larvae had an average snout-vent length of 2.18 (± 0.05) cm and total length of 6.01 (± 0.14) cm and were used at Gosner stage 26-30 which is characterized by rapid growth and development of hind limbs (pro-premetamorphic larvae, based on the developmental table of Gosner (1960)). Tadpoles at this stage were selected for the MN test because they are large enough to allow for cardiac puncture and provide a sufficient amount of blood for smears. Additionally, intense hematopoiesis occurs during this period with active cell division in circulating blood (Deparis, 1973). These peripheral erythrocytes are, therefore, suitable for MN detection when cells are readily scored from blood smears.

2.3 - Tadpole care

Tadpoles were reared in the aquatics facility in the VCU biology department (Room 045, Trani Center for Life Sciences) in compliance with IACUC protocol (#AD10000012) and contained within 15L of water in 10 gallon Perfecto® aquaria, at densities of 3 tadpoles per aquarium, and with ultrapurified water at 21° C. All tanks were covered with glass lids to prevent contamination of samples. Water was agitated and aerated by bubbling, and was filtered using Hydro Pro Sponge filters. Tadpoles were fed daily, with commercial tadpole food (Carolina Biological Supply Company [item #146500]) and excess food was removed each day prior to feedings.

Complete water changes were performed every four days to maintain effective concentrations of the test compounds. A random sample of tanks was tested daily before feedings for levels of ammonia, nitrates and nitrites using Mardel® Master Kit chromo-metric test strips. At no point during experimentation did any tank register levels of these cycling products detectable by the chromo-metric test strips. The pH of water was also monitored using chromo-metric strips and was maintained at 7.0.

2.4 - Preparation of Chemical Concentrations

Stock solutions were prepared to simplify chemical additions during water changes. Cyclophosphamide (2.5g) was completely dissolved (solubility = 40g/L) in 500 mL of ultrapurified water to yield a concentration of 5g/L. At each water change for the CYP treatments, 15mL of the stock solution was added to 15L of fresh water in the tank to yield a final treatment concentration of 5mg/L (5ppm). TCS (138mg) was completely dissolved

in 20L (solubility = 0.012g/L) of ultrapurified water to yield a stock concentration of 6.9µg/mL. At each water change, either 500mL; 50mL; or 5mL of this stock solution was added to ultrapurified water in each TCS trial tank to yield treatment concentrations of 0.23mg/L; 0.023mg/L; and 0.0023mg/L, respectively.

Tadpoles were placed three-per-tank and a single tadpole was removed from each tank after 1, 8, and 15 days following initial exposure to the compounds (8 tadpoles per level at each endpoint). There were 24 tadpoles in each treatment group (Figure 4).

2.5 - Blood smear preparation and analysis

At each endpoint, tadpoles were placed in labeled cups filled with uncontaminated water and were taken to Sanger Hall, where each tadpole was euthanized in MS-222 and photographed to allow for later measurement using ImageJ software. Following photography, the tadpoles were exsanguinated via cardiac puncture. Blood from each tadpole was smeared onto two clean microscope slides which were air dried overnight. The slides were then immersed in Carnoy's fixative (3 parts methanol: 1 part glacial acetic acid) for 10 minutes, immediately stained with Giemsa solution for 10 minutes following fixation, and then briefly rinsed under gentle tap water to remove excess Giemsa solution. Slides were air dried and stored at room temperature until evaluated. The MN frequency was assessed in 1000 cells per slide (2000 erythrocytes per animal were scored) and the number of cells containing one or more MN was recorded under 1,000x magnification using a Zeiss[®] Axioskop 2 microscope. Slides were alpha-numerically coded and

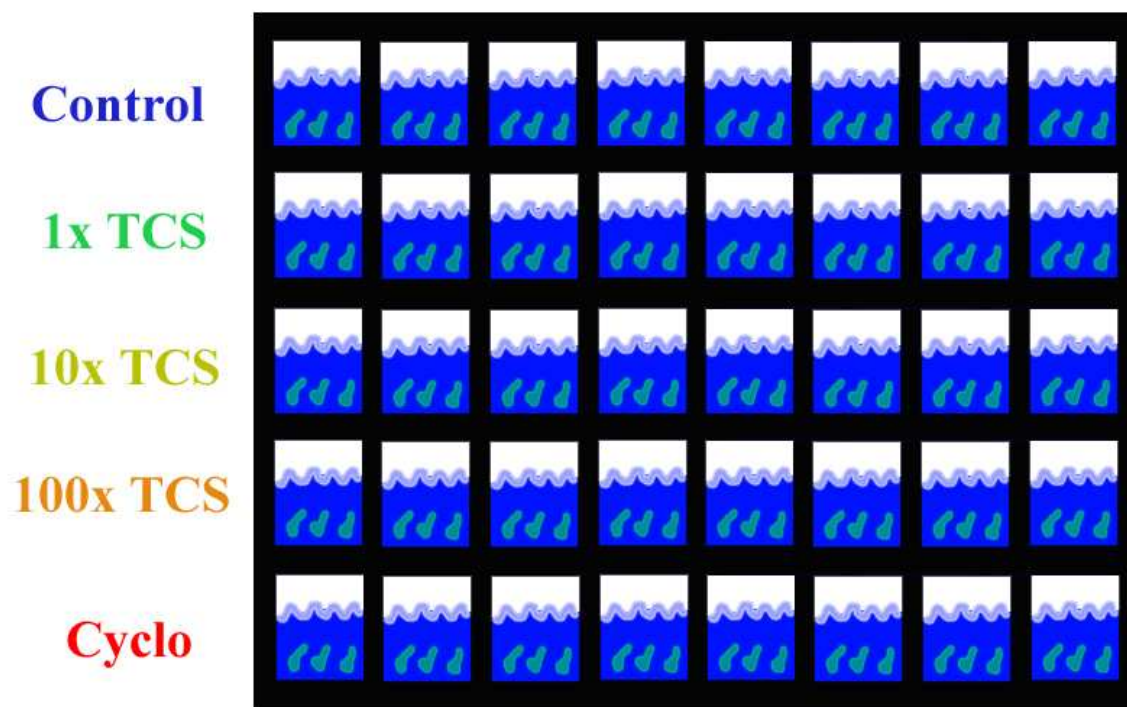


Figure 4 – Experimental design. Tadpoles were placed three-per-tank and a single tadpole was removed from each tank after 1, 8 or 15 days following initial exposure to test compounds.

haphazardly selected for scoring by a single, blinded observer. MN counts were expressed as sum total of the MN scored between two slides per animal.

2.6 - Statistical analyses

Micronucleus count data were found to be non-normally distributed, so count data were log-transformed before analysis. One-way ANOVAs were performed on the response variables of MN counts and size effects by treatment and time to test for significant differences between treatment groups on induction of micronucleated cells. A direct linear relationship did not best describe the effects of time and treatment on MN counts, so stepwise linear mixed-effect models were created in R to test the main effects of treatment, time, and a second order polynomial of time for MN counts by treatment and by TCS concentration. Models were selected based on Akaike's information criterion (AIC) values (Akaike, 1992), where lower values designate better fits, using the 'nlme' package within R statistical software. 'Sampled tank' was treated as a random effect due to tank resampling across time endpoints. The 'glht' command within the multcomp package of R was used to test pairwise comparisons between treatments and Tukey post-hoc analyses (Bonferroni) were performed to quantify two-tailed differences between groups, where appropriate. Significant differences were tested against $\alpha=0.05$.

CHAPTER 3

Results

3.1 - Micronucleus Induction Counts

Average MN counts were expressed as number of cells containing one or more micronuclei per 2000 red blood cells scored ($\pm 1SE$) and are summarized in Table 1. Differences between counts of micronucleated erythrocytes by treatment are visually presented in Figure 5, and differences between numbers of micronucleated erythrocytes by treatment and time are presented in Figure 6. Total number of tadpoles scored was $n=23$ in each of the control, 1x TCS, 10x TCS, and CYP treatments and $n=24$ in the 100x TCS treatment. Erythrocytes from three tadpoles (one from control @ 24h, one from 1x TCS @ 24h, one from Cyclo @ 168h treatments) were unable to be scored for MN presence on either slide for the animal, perhaps due to contamination of the sample by skin mucus or staining error. Seven slides were randomly recounted to test for counting error and this source of error was determined to be insignificant. A single fatality occurred in the 10x TCS @ 168h treatment; erythrocytes from the deceased animal were unable to be scored.

Micronucleus count data were tested for normality using the Shapiro-Wilk normality test and were found to be non-normally distributed. Therefore, count data were log-transformed for subsequent analysis. Analysis of variance of count data by treatment yielded a significant effect of treatment ($F=13.71$, $p=5.12e-09$, $df=4$), of time ($F=40.639$, $p=5.112e-09$, $df=1$), and of a time² term ($F=35.783$, $p=3.16e-08$, $df=1$) on MN frequency.

Table 1 – Frequency of micronucleated red blood cells (per 2000 cells) in larvae treated with different concentrations of test compounds. Count data is represented as mean \pm 1 Standard Error.

Treatment	Concentration	Total # cells scored (= $n_{\text{radpoles}} \times 2000$)	# MN cells	# MN cells/ 2,000 erythrocytes (mean \pm SE)			
				Total	24h	168h	336h
Control	0	46,000	38	1.65 (± 0.32)	0.42 (± 0.20)	2.75 (± 0.56)	1.63 (± 0.48)
1xTCS	2.3 $\mu\text{g/L}$	46,000	134	5.83 (± 0.64)	2.86 (± 0.40)	6.875 (± 1.16)	7.38 (± 0.78)
10xTCS	23 $\mu\text{g/L}$	46,000	199	8.65 (± 0.89)	5.00 (± 0.53)	10.29 (± 1.48)	10.88 (± 1.52)
100xTCS	230 $\mu\text{g/L}$	48,000	269	11.21 (± 0.93)	7.88 (± 0.64)	15.00 (± 1.75)	10.75 (± 1.16)
CYP	5 mg/L	46,000	318	13.83 (± 1.2)	8.88 (± 1.125)	20.71 (± 1.01)	12.75 (± 1.18)

There was no significant interaction of treatment and time ($F=1.250$, $p=0.29$, $df=4$) so this interaction was dropped from subsequent analysis.

3.2 - Statistical Analysis by Treatment

A stepwise linear mixed-effects model was created and each model was tested for fit in a stepwise manner, where the terms of treatment, time, and $time^2$ were added and removed from the model until the best (lowest) Akaike information criterion (AIC) value was obtained (Akaike, 1992). A model lower than another by more than 2 AIC units was considered to be more significantly supported (Burnham and Anderson, 1998). The best model retained the terms of treatment, time, and $time^2$ in the model (AIC=105.8). Multiple comparisons of means were conducted via Tukey post-hoc contrasts with reported p-values adjusted by the Bonferroni method. With regard to the best-fit model, it was determined that MN counts for the negative control treatments were significantly lower than the 1x TCS treatment ($z=-9.81$, $p<2e-16$), lower than the 10x TCS treatment ($z=-13.55$, $p<2e-16$), lower than the 100x TCS treatment ($z=-16.154$, $p<2e-16$), and lower than the positive control ($z=-17.79$, $p<2e-16$). The 1x TCS treatment was significantly lower than the 10x TCS treatment ($z=-3.75$, $p=1.76e-03$), significantly lower than the 100x treatment ($z=-6.25$, $p=4.21e-09$), and significantly lower than the CYP treatment ($z=-7.99$, $p=1.33e-14$). The 10x TCS treatment did not differ significantly from the 100x TCS treatment ($z=-2.454$, $p=0.14$), but was significantly lower than the positive control ($z=-4.24$, $p=2.23e-4$). The 100x TCS treatment did not differ significantly from the CYP treatment ($z=-1.83$, $p=0.67$)(Figure 5).

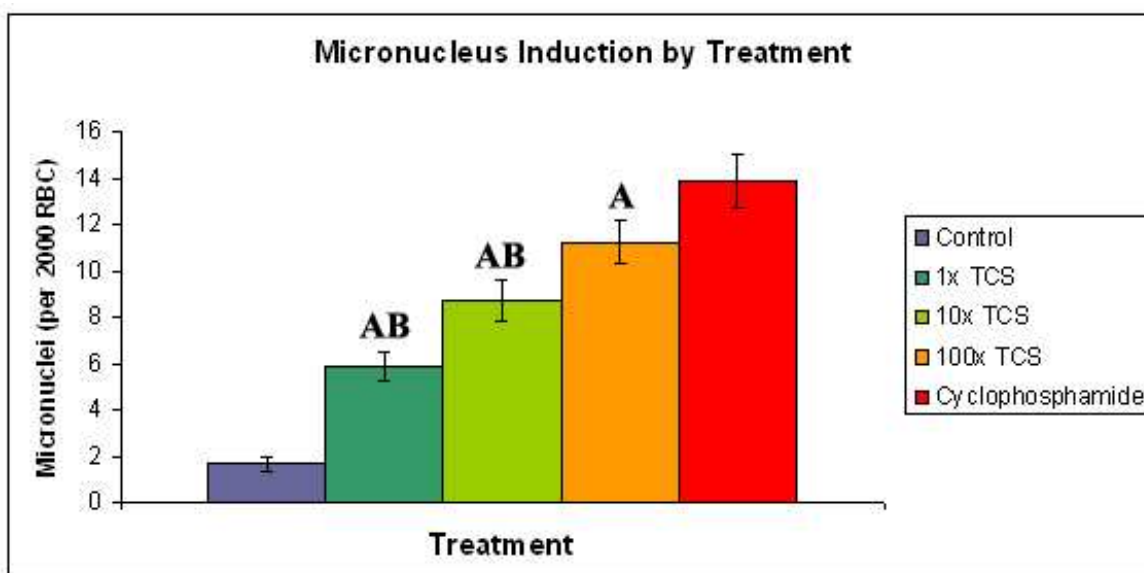


Figure 5 – Micronucleus Induction by Treatment. This figure depicts cumulative number of erythrocytes containing at least one MN per 2000 erythrocytes scored, compiled from all three endpoints. (A) denotes significant difference from the negative control ($p < 0.001$) and (B) denotes significant difference from the positive control ($P < 0.001$). Error bars represent ± 1 Standard Error.

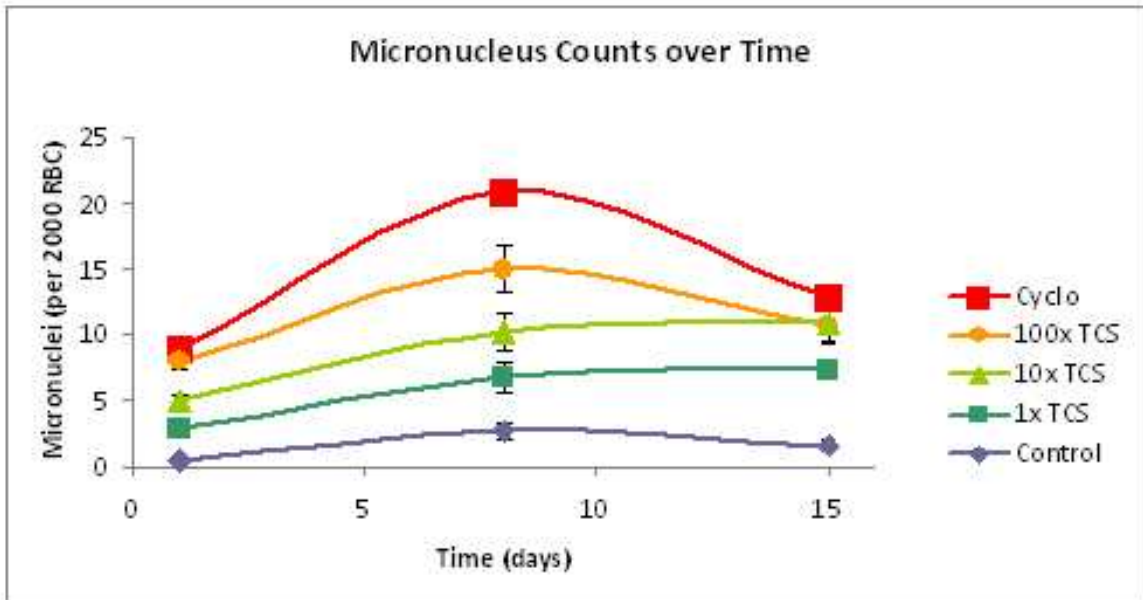


Figure 6 – Micronucleus induction by treatment over time. This figure depicts the number of erythrocytes containing at least one MN per 2000 erythrocytes scored, separated by endpoint. Note the negative binomial hump-shaped curve for each treatment examined through model analysis. Error bars represent ± 1 Standard Error.

The CYP treatment was removed from subsequent analysis in order to best test models of MN induction by TCS. A significant hump-shaped curve of MN frequency can be seen in Figure 6 for each treatment, as described by a negative binomial term of time². The model was tested through a stepwise linear mixed-effects model where the terms of treatment, time, and time² were added and removed in a stepwise manner to achieve the maximum likelihood of fit as designated by Akaike information criterion (AIC) values. Lower AIC values designate a better-fit model. The best model was selected and included all three terms (AIC=96.02). Removal of the time² term increased the AIC value to 113.65, so the model including treatment, time, and time² was significantly better.

3.3 - Micronucleus Induction via Dose-Dependence

An analysis of variance of MN count data by TCS concentration found a significant effect of concentration (F=5.01, p=0.03, df=1), of time (F=23.29, p=5.89e-06, df=1), and a significant time² term (F=15.288, p=1.83e-04, df=1). There was no significant interaction of concentration and time (F=1.84, p=0.18, df=1). Linear mixed-effect models were applied to each treatment, independently, to analyze differences in model shape between treatment groups. Terms α , β , γ were extracted from the model $\log(\text{MN}+1)=\alpha*\text{time} + \beta*\text{time}^2 + \gamma$ for each treatment and were compared using 95% confidence intervals calculated using a t critical value of $t(13)=2.160$ for control, 1x TCS, and 10x TCS with df=13 in each model and a t critical value of $t(14)=2.145$ for the 100x TCS concentration model with df=14. Confidence intervals overlapped for all treatments for terms α and β , but not for γ . Significant differences in the intercept term γ were found between negative

control and all TCS treatments and between the 1x and 100x TCS treatments, suggesting that MN induction is dose-dependent (Figure 7).

Micronucleus induction was confirmed to be TCS dose-dependent as described by the formula $\log(\text{MN}+1)+1 = 2.059(\text{CONC})^{0.0179}$. This formula was determined by a significant power regression ($p < 0.000$, $r^2 = 0.62$) (Figure 8).

3.4 - Length Effects of Treatment

Change in snout-vent length (SVL) and total length (TL) were tested using a Shapiro-Wilk normality test and were found to be normally distributed. Analysis of variance of treatment effect on change in SVL found no significant differences ($F = 0.11$, $p = 0.98$, $df = 4, 115$) (Figure 9). Analysis of variance of treatment effect on change in TL found no significant differences ($F = 0.31$, $p = 0.887$, $df = 4, 115$). A subset of tadpoles removed at the 15 day endpoint was analyzed separately and was found to be distributed normally. Analysis of variance of this subset also failed to reveal any significant differences between treatment and change in total length ($F = 0.35$, $p = 0.84$, $df = 4, 35$) (Figure 10).

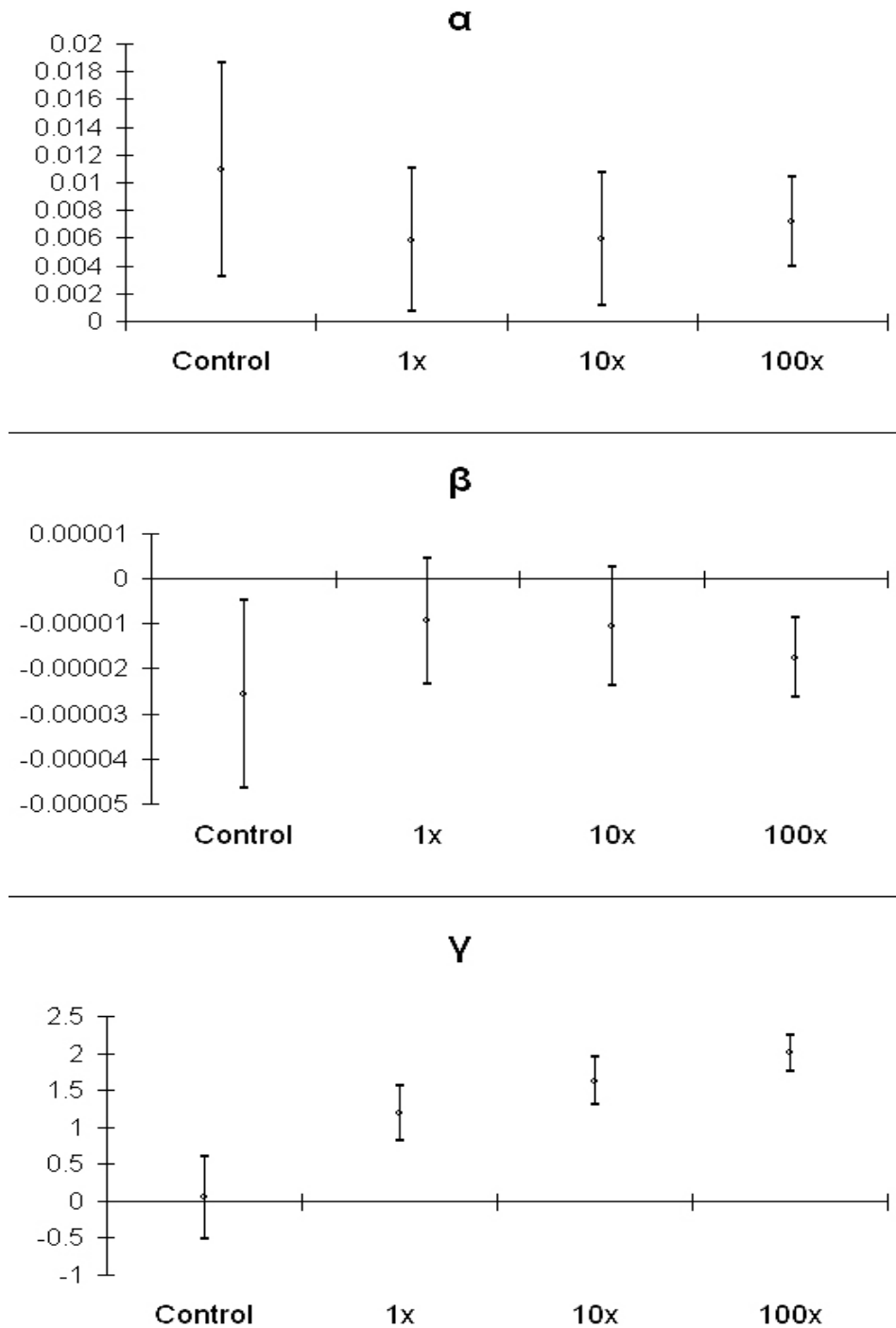


Figure 7 – Parameter estimates and 95% confidence intervals for polynomial descriptive terms α , β , γ for TCS treatments, as described by the generalized mixed effects model with formula $\log(MN+1)=\alpha*\text{time} + \beta*\text{time}^2 + \gamma$.

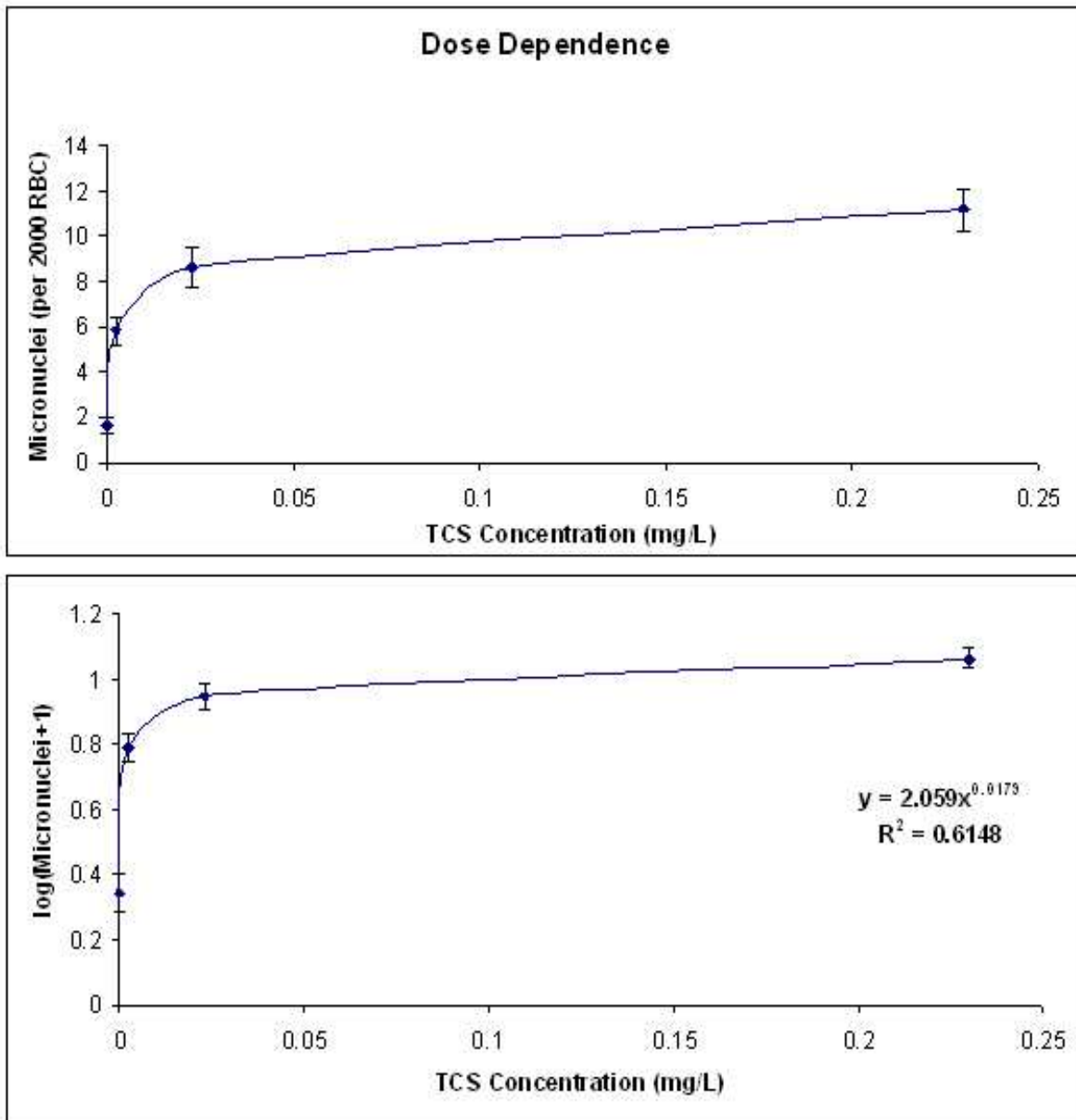


Figure 8 – Dose dependent curve reflecting the relationship between TCS concentration and micronucleated cell counts. Error bars represent ±1 Standard Error.

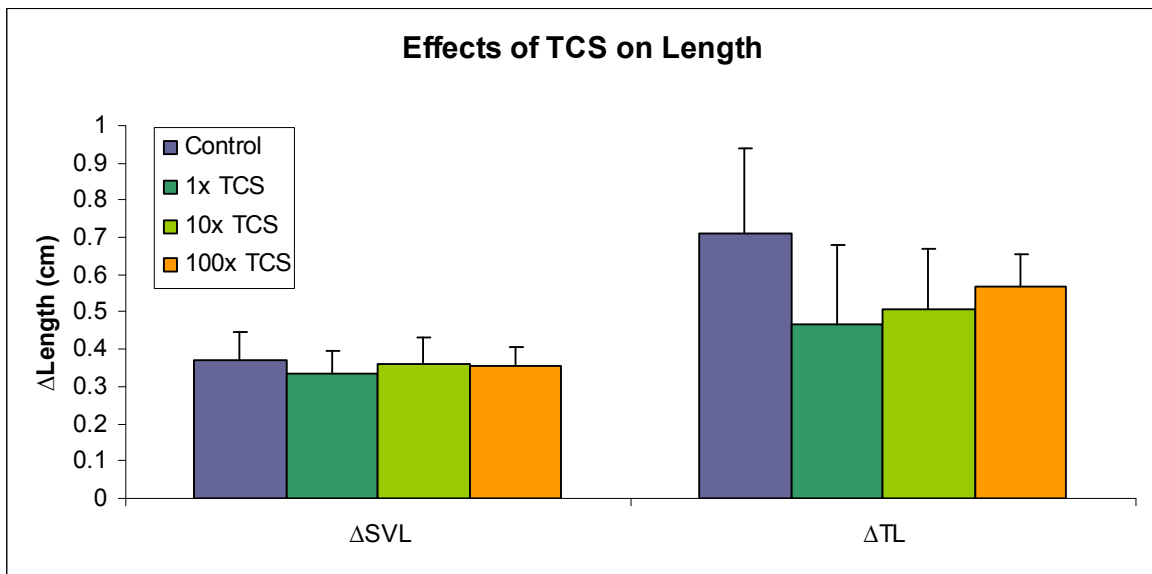


Figure 9 – Change in Snout-vent length (SVL) and Total length (TL) of tadpoles in cm over the study period by treatment. Error bars represent ± 1 Standard Error.

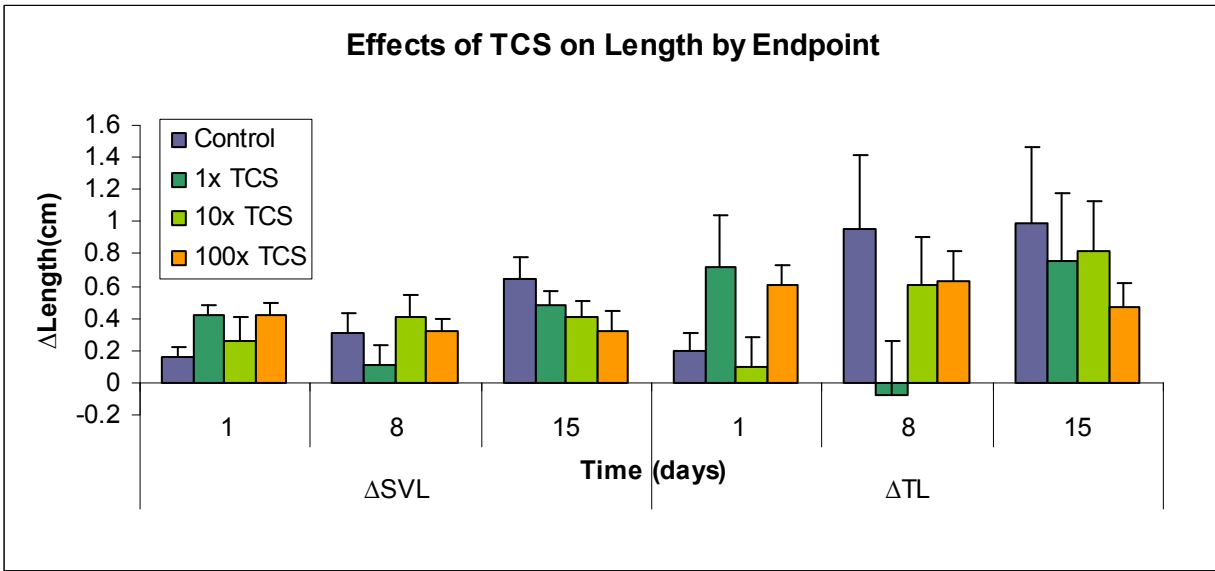


Figure 10 – Change in Snout-vent Length (SVL) and Total Length (TL) of tadpoles in cm over the study period by endpoint. Error bars represent ± 1 Standard Error.

CHAPTER 4

Discussion

4.1 - The Genotoxicity of Triclosan to *Lithobates catesbeianus*

All TCS treatments induced MN counts significantly greater than the negative control suggesting that TCS is genotoxic to *Lithobates catesbeianus*. At concentrations of TCS similar to those found in US surfacewaters, 2.3µg/L, treatment yielded significantly greater MN induction than pure water. The highest concentration (100x) of TCS induced MN counts not significantly different from a known genotoxin, CYP, further supporting that TCS can cause significant genotoxic damage. The maximum induction by TCS was about 15 micronuclei per 2000 erythrocytes and was achieved between 8 and 15 days by treatment with 230µg/L TCS. Testing daily endpoints between 8 and 15 days would help resolve this maximum of induction.

It is important to note that the micronucleus counts for these tadpoles included cells that may have already exhibited micronucleus presence prior to Triclosan administration. *In vitro* cells are often treated with cytochalasin B prior to micronucleus scoring so that any cells undergoing cell division are unable to complete cytoplasmic division. In this method, *in vitro* cells can be scored by only scoring binucleated cells for micronucleus presence. In the present study, examining erythrocytes *in vivo* does not allow for cytochalasin B treatment, so micronuclei appearing before treatment would also be counted. While this is a limitation of testing micronucleus presence *in vivo*, comparison to

the controls shows a clear difference between animals treated with Triclosan and those untreated.

4.2 - Modeling Genotoxicity

Modeling the parameters α , β , and γ , which mathematically define the curve fitting the counts of micronuclei over time by treatment, suggests that lower concentrations (1x, 10x) would likely reach a similar maximum of induction as the 100x TCS treatment and that the best predictor of curve fit would be the y-intercept term, γ . In this model, γ is a representation of the acute genotoxicity induced by each level of treatment, or the MN induction noted at (or before) the 24-hour endpoint. Significant differences in MN induction as described by this intercept variable support that MN induction by TCS is dose-dependent. Overlapping of the 95% confidence intervals of the coefficients α and β suggests that an absence of significant difference exists between each of these coefficients by treatment, which supports that the shape of the curves over time is not significantly affected by the coefficients α and β . This suggests that the mechanism of MN induction does not differ by treatment; that only the magnitude of initial induction differs as described by γ . Mechanistically, this second-order polynomial phenomenon could possibly be explained by MN clearing, among other probable mechanisms for the noted reduction. As micronuclei accumulate, apoptosis is triggered by normal cell defenses, leading to the elimination of cells with premutagenic/mutagenic lesions. This mechanism of cell clearing would decrease the proportion of micronucleated cells over time. The increase in apoptotic cell fractions, as corresponding to a decrease in MN frequency, could

be tested by analysis of induction of apoptosis using magnetic annexin microbead cell sorting (the annexin-V test) (Decordier et al., 2002).

Studies on *Lithobates catesbeianus* tadpoles suggest that erythrocytes mature in the liver in the tadpole and in the bone marrow of adult frogs (Maniatis and Ingram, 1971). The erythropoiesis process occurs at two sites, the kidney and the liver. Broyles et al. (1981) have found that there are two morphologically different types of erythrocytes, originating in the liver or kidney, which seem to correspond to a switch in hemoglobin type as the tadpole prepares for metamorphosis. While some investigators have reported an average erythrocyte life span of 98.7 days in young tadpoles (Forman and Just, 1976), other studies have found much shorter life spans in their research, such as 24 days in *L. catesbeianus* (Baca Saravia, 1961). Forman and Just (1976) concluded that during the transition from tadpole to frog, the tadpole erythrocyte life span must be drastically shortened to account for the hemoglobin transition observed during amphibian metamorphosis. Furthermore, Tamori and Wakahara (2000) found that mature larval-type erythrocytes are specifically removed from metamorphosing *Xenopus* individuals via apoptosis, a process which is driven by thyroid hormones. It is likely, therefore, that the premetamorphic tadpoles used in this study were exhibiting greater generation of adult erythrocytes during experimentation than younger tadpoles would exhibit, which may account for such dramatic acute TCS genotoxicity as shown by high micronucleus counts.

Veldhoen (2006) demonstrated that TCS altered thyroid hormone (TH) receptor α transcript levels in the brain of tadpoles and that exposure to concentrations of TCS as low as 0.03 $\mu\text{g/L}$ for 24 hours altered thyroid hormone receptor mRNA expression in *Rana*

catesbeiana. This lead to earlier metamorphosis of the exposed tadpoles and would likely, therefore, increase the turnover of erythrocytes as well. This effect of TCS may be particularly damaging as TCS is being removed by the liver of exposed animals, which is also the main organ that activates TH, turning the T4 produced by the thyroid into active T3. Via this mechanism, TCS may be significantly more genotoxic to the erythrocytes of premetamorphic tadpoles close to metamorphosis than younger tadpoles exhibiting the longer-lived erythrocyte morphology.

4.3 - Size Effects

There was no significant effect of treatment on differences in SVL and TL between the beginning of the experiment and each endpoint. While Veldhoen (2006) demonstrated that TCS altered thyroid hormone receptor α transcript levels in the brain of tadpoles and induced a transient weight loss and since TCS leads to early metamorphosis, one might expect that increasing concentrations of TCS might induce increasingly larger reductions in total length change. An effect of TCS on SVL or TL change was not significant in this study over 15 days, nor was it significant in Veldhoen's study over 18 days. While Veldhoen did note a sudden increase in tadpole weight after administration of TCS, it is likely that no significant detectable length changes would be observable in 15-18 days for a tadpole which takes over a year to achieve metamorphosis.

4.4 - The Need for Regulation

The concentration-dependent induction of micronuclei examined in this study suggests that an increase in TCS concentrations will cause more genotoxicity, but the comparative effect of ten-fold concentration increases beyond 23 μ g/L would begin to diminish. That is to say, perhaps, that the environmental concentrations of TCS in surface waters now (at a maximum between 2.3 μ g/L (Kolpin et al., 2002) and 2.7 μ g/L (Reiss et al., 2002) are at a point where regulatory management would make a significant difference in the physiological effects of the compound on wildlife.

While this study examined the genotoxic response of *Lithobates catesbeianus* to prolonged exposure to TCS, it would be interesting to directly assess the genotoxic effects of a single pulse of TCS at different concentrations over time. It is likely that animals exposed to a single low dose of TCS would return to baseline MN counts after a period of time. While this study may support that proper regulatory action can alleviate the physiological stressors to aquatic organisms imposed by TCS over time, it is unclear how long it would take for damaging effects on wildlife to be remedied. Though TCS has an estimated half-life of 60 days in water (Halden and Paul, 2005), methyl-TCS has been found to be more persistent in aquatic systems and has a greater lipophilicity to aquatic organisms (Coogen et al., 2007). It is clear that more studies need to be conducted on the effects of methyl-TCS, its super-chlorinated dioxin congener, and combinatorial effects of TCS and other chemical toxins need to be studied *in vivo* and *in situ*.

Pharmaceuticals and personal care products are, by design, hazardous to biological systems and the normal development of wildlife species and humans. Investigators have

identified TCS as an environmental pollutant with the ability to bioaccumulate in fish at sublethal concentrations (Adolfsson-Erici et al., 2002; Orvos et al., 2002; Balmer et al., 2004; and Valters et al., 2005), act as a direct toxicant in different aquatic species and at different life stages such as algae (Wilson et al., 2003; Orvos et al., 2002); water fleas, rainbow trout (Orvos et al., 2002); and medaka (*Oryzias latipes*) (Ishibashi et al., 2004). Triclosan induces species-specific mortality in four species of frogs, *Acris crepitans blanchardii*, *Bufo woodhousii woodhousii*, and *Rana sphenoccephala*, and *Xenopus laevis* as designated by LC50 values between 152µg/L and 664µg/L (Palenske et al., 2010); increased LC50 values appear to correlate directly with aquatic period of the frog across life stages. This may seem slightly counterintuitive, but it appears that the tolerance of the most aquatic frogs to TCS is greater than that of toads and tree frogs, which spend less time in an aquatic environment. In line with this observation, it should be noted that American toad (*Bufo americanus*) tadpoles exposed to the same 2.3 µg/L concentration used in this study (1x TCS) showed significantly higher mortality than controls (Smith and Burgett, 2005). In contrast, only one fatality was observed in *Lithobates catesbeianus* at the 10xTCS (23µg/L) concentration in this study. It would follow that *Lithobates catesbeianus*, which is aquatic or semiaquatic throughout its entire life cycle, would have a similar TCS LC50 as *R. sphenoccephala*, if not greater due to increased larval period in *L. catesbeianus*. *Lithobates catesbeianus* would, therefore, be an extremely appropriate model for examining the sublethal or cumulative effects of Triclosan, and perhaps other environmental contaminants *in vivo* and *in situ*.

The lipophilic nature of TCS poses additional threats to wildlife when bioconcentration occurs and the compound becomes concentrated in tissues of organisms at higher trophic levels. Blood plasma collected from wild Atlantic bottlenose dolphins (*Tursiops truncatus*) in South Carolina and Florida ranged from 0.072ng/g wet tissue weight to 0.27ng/g, with 23% and 31% of the sampled individuals from the two locations showing detectable levels of Triclosan (Fair et al., 2009). It is therefore likely that TCS is, as have many persistent organic pollutants, beginning to biomagnify in waterfowl, wading birds, raptors, and fish-eating mammals. Humans are directly exposed to concentrations of TCS in PCPPs far exceeding those of surfacewaters and are likely at higher risk than other vertebrates.

4.5 - Future Directions

The utility of the MN bioassay extends beyond assessing solely genotoxicity *in vivo*. In a study where micronuclei were induced in mice with the drug vinblastine, acute stress-induced mice (via restraining) showed significantly higher MN counts than the unstressed mice (Malvandi et al., 2010). This combinatorial effect of stress and chemical induction of micronuclei suggests that the MN bioassay may be useful as a proxy of ecological stress *in situ*, by comparing MN counts between similar or the same species from different sites and testing for correlation between MN counts and other sources of environmental stress. For example, a study which compares MN counts of *Lithobates catesbeianus* tadpoles from sites proximal to centers of human population density, agricultural land, industrial facilities, or impervious surfaces may find a correlation

between MN counts and disturbance. This would allow for environmental surveys to be conducted using MN frequencies as proxies for ecosystem health, which then could be extrapolated and related to historical human health trends or trends in wildlife declines or environmental degradation. *Lithobates catesbeianus* would be an obvious model choice for said study.

It would also be helpful to analyze the literature of studies pertaining to micronucleus induction following exposure to genotoxins in order to gauge the relative risk of exposure to Triclosan as compared to other genotoxic inducers. Using a proportional analysis by comparing control micronucleus counts to post-treatment counts would give an approximation of effect size of a given genotoxin allowing for such a meta-analysis. In this way, we could approximate relative risk of different genotoxins in vertebrate models.

It is clear that the effects of genotoxic agents must be certified so proper regulatory protocols can be developed and enforced in order to better conserve wildlife and promote human health; more conservation-medicine disciplined studies should be conducted to better correlate zoonotic diseases, wildlife stressors, and ecosystem degradation with human health risks.

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Appendix A – R scripts and outputs

```
> library(RODBC)
> channel<-odbcConnectExcel("C:/TriclosanData.xls")
> TRICLO<-sqlFetch(channel, "Sheet1")
> odbcClose(channel)
> TRICLO1.aov<-aov(log(MICRO+1) ~ TTT * TIME +I(TIME^2)+ Error(TK),
data=TRICLO)
> summary(TRICLO1.aov)
```

Error: TK

	Df	Sum Sq	Mean Sq
TTT	1	41.074	41.074

Error: Within

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
TTT	4	7.2769	1.8192	13.713	5.152e-09 ***
TIME	1	5.3914	5.3914	40.639	5.118e-09 ***
I(TIME^2)	1	4.7472	4.7472	35.783	3.156e-08 ***
TTT:TIME	4	0.6633	0.1658	1.250	0.2946
Residuals	104	13.7972	0.1327		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> NOCYCLO<-subset(TRICLO, TTT %in% c("LOW","MED","HIGH","NEG_CTL"))
> TRICLO2.aov<-aov(log(MICRO+1) ~ CONC * TIME + I(TIME^2) + Error(TK),
data=NOCYCLO)
> summary(TRICLO2.aov)
```

Error: TK

	Df	Sum Sq	Mean Sq
CONC	1	30.959	30.959

Error: Within

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
CONC	1	0.9933	0.9933	5.0060	0.0278140 *
TIME	1	4.6220	4.6220	23.2936	5.89e-06 ***
I(TIME^2)	1	3.0317	3.0317	15.2793	0.0001831 ***
CONC:TIME	1	0.3650	0.3650	1.8396	0.1785077
Residuals	87	17.2627	0.1984		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```
> lme_micro <-stepAIC(lme(log(MICRO+1) ~ TTT*TIME +I(TIME^2),
na.action=na.omit, data=NOCYCLO, method="ML", random =~1|TK))
Start: AIC=97.95
log(MICRO + 1) ~ TTT * TIME + I(TIME^2)
```

```
      Df  AIC
- TTT:TIME  3 96.017
<none>      97.949
- I(TIME^2)  1 116.522
```

```
Step: AIC=96.02
log(MICRO + 1) ~ TTT + TIME + I(TIME^2)
```

```
      Df  AIC
<none>      96.017
- I(TIME^2)  1 113.649
- TIME       1 124.309
- TTT        3 164.099
```

```
> summary(lme_micro)
Linear mixed-effects model fit by maximum likelihood
Data: NOCYCLO
      AIC   BIC logLik
96.01697 116.2778 -40.00849
```

```
Random effects:
Formula: ~1 | TK
      (Intercept) Residual
StdDev: 1.863571e-05 0.3720453
```

```
Fixed effects: log(MICRO + 1) ~ TTT + TIME + I(TIME^2)
      Value Std.Error DF  t-value p-value
(Intercept) 1.8608303 0.11188010 59 16.632363 0.0000
TTTLOW      -0.6442201 0.11226971 28 -5.738147 0.0000
TTTMED      -0.2543463 0.11226926 28 -2.265503 0.0314
TTTNEG_CTL -1.6658207 0.11226971 28 -14.837668 0.0000
TIME         0.0074730 0.00129119 59  5.787663 0.0000
I(TIME^2)   -0.0000158 0.00000350 59 -4.521987 0.0000
```

```
Correlation:
      (Intr) TTTLOW TTTMED TTTNEG TIME
TTTLOW  -0.476
TTTMED  -0.502   0.489
TTTNEG_CTL -0.476  0.490   0.489
TIME     -0.602  -0.015   0.021  -0.015
```

I(TIME^2) 0.492 0.010 -0.022 0.010 -0.970

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.81198032	-0.67350578	0.01550276	0.81367923	1.85702156

Number of Observations: 93

Number of Groups: 32

```
> lme_micro <- stepAIC(lme(log(MICRO+1) ~ TTT*TIME + I(TIME^2),  
na.action=na.omit, data=TRICLO, method="ML", random =~1|TK))
```

Start: AIC=108.33

```
log(MICRO + 1) ~ TTT * TIME + I(TIME^2)
```

	Df	AIC
- TTT:TIME	4	105.81
<none>		108.33
- I(TIME^2)	1	140.77

Step: AIC=105.81

```
log(MICRO + 1) ~ TTT + TIME + I(TIME^2)
```

	Df	AIC
<none>		105.81
- I(TIME^2)	1	136.64
- TIME	1	150.32
- TTT	4	197.64

```
> summary(lme_micro)
```

Linear mixed-effects model fit by maximum likelihood

Data: TRICLO

	AIC	BIC	logLik
	105.8113	130.5936	-43.90566

Random effects:

Formula: ~1 | TK

(Intercept) Residual

StdDev: 1.534969e-05 0.3532987

Fixed effects: log(MICRO + 1) ~ TTT + TIME + I(TIME^2)

	Value	Std.Error	DF	t-value	p-value
(Intercept)	1.8525713	0.10045178	74	18.442395	0.0000
TTTLOW	-0.6440171	0.10637049	35	-6.054472	0.0000
TTTMED	-0.2529972	0.10637049	35	-2.378453	0.0230

```

TTTNEG_CTL -1.6656177 0.10637049 35 -15.658644 0.0000
TTTPOS_CTL 0.1887597 0.10637049 35 1.774549 0.0847
TIME 0.0080593 0.00109912 74 7.332481 0.0000
I(TIME^2) -0.0000178 0.00000298 74 -5.971513 0.0000

```

Correlation:

```

(Intr) TTTLOW TTTMED TTTNEG TTTPOS TIME
TTTLOW -0.505
TTTMED -0.528 0.489
TTTNEG_CTL -0.505 0.490 0.489
TTTPOS_CTL -0.528 0.489 0.490 0.489
TIME -0.568 -0.013 0.019 -0.013 0.019
I(TIME^2) 0.465 0.009 -0.020 0.009 -0.020 -0.971

```

Standardized Within-Group Residuals:

```

      Min      Q1      Med      Q3      Max
-3.04958949 -0.70504653 0.03581990 0.80719676 2.05602285

```

Number of Observations: 116

Number of Groups: 40

```

> summary(glht(lme_micro, linfct=mcp(TTT = "Tukey")), test = adjusted(type =
"bonferroni"))

```

Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

```

Fit: lme.formula(fixed = log(MICRO + 1) ~ TTT + TIME + I(TIME^2),
data = TRICLO, random = ~1 | TK, method = "ML", na.action = na.omit)

```

Linear Hypotheses:

	Estimate	Std. Error	z value	Pr(> z)
LOW - HIGH == 0	-0.6440	0.1031	-6.246	4.21e-09 ***
MED - HIGH == 0	-0.2530	0.1031	-2.454	0.141420
NEG_CTL - HIGH == 0	-1.6656	0.1031	-16.154	< 2e-16 ***
POS_CTL - HIGH == 0	0.1888	0.1031	1.831	0.671538
MED - LOW == 0	0.3910	0.1042	3.751	0.001761 **
NEG_CTL - LOW == 0	-1.0216	0.1042	-9.806	< 2e-16 ***
POS_CTL - LOW == 0	0.8328	0.1042	7.989	1.33e-14 ***
NEG_CTL - MED == 0	-1.4126	0.1042	-13.551	< 2e-16 ***
POS_CTL - MED == 0	0.4418	0.1042	4.240	0.000223 ***
POS_CTL - NEG_CTL == 0	1.8544	0.1042	17.789	< 2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
(Adjusted p values reported -- bonferroni method)

```
> POS_CTL<- subset(TRICLO, subset=TTT=="POS_CTL")
> NEG_CTL<- subset(TRICLO, subset=TTT=="NEG_CTL")
> NEG_CTL<- subset(TRICLO, subset=TTT=="NEG_CTL")
> HIGH<- subset(TRICLO, subset=TTT=="HIGH")
> MED<- subset(TRICLO, subset=TTT=="MED")
> LOW<- subset(TRICLO, subset=TTT=="LOW")
```

```
> lme_NEG_CTL<-lme(log(MICRO+1)~TIME+I(TIME^2), na.action=na.omit,
data=NEG_CTL, method="ML", random =~1|TK)
```

```
> summary(lme_NEG_CTL)
```

Linear mixed-effects model fit by maximum likelihood

Data: NEG_CTL

	AIC	BIC	logLik
	43.23443	48.9119	-16.61722

Random effects:

Formula: ~1 | TK

(Intercept) Residual

StdDev: 2.401149e-05 0.4983509

Fixed effects: log(MICRO + 1) ~ TIME + I(TIME^2)

	Value	Std.Error	DF	t-value	p-value
--	-------	-----------	----	---------	---------

(Intercept)	0.04930691	0.25719117	13	0.1917131	0.8509
-------------	------------	------------	----	-----------	--------

TIME	0.01094027	0.00358761	13	3.0494567	0.0093
------	------------	------------	----	-----------	--------

I(TIME^2)	-0.00002571	0.00000967	13	-2.6585797	0.0197
-----------	-------------	------------	----	------------	--------

Correlation:

(Intr) TIME

TIME	-0.759
------	--------

I(TIME^2)	0.616	-0.969
-----------	-------	--------

Standardized Within-Group Residuals:

	Min	Q1	Med	Q3	Max
	-2.4119064	-0.5960922	0.3698572	0.8062054	1.5792718

Number of Observations: 23

Number of Groups: 8

```
> lme_LOW<-lme(log(MICRO+1)~TIME+I(TIME^2), na.action=na.omit, data=LOW,
method="ML", random =~1|TK)
```

```
> summary(lme_LOW)
```

Linear mixed-effects model fit by maximum likelihood

Data: LOW

AIC BIC logLik
24.66433 30.3418 -7.332166

Random effects:

Formula: ~1 | TK

(Intercept) Residual

StdDev: 8.245336e-05 0.3328216

Fixed effects: $\log(\text{MICRO} + 1) \sim \text{TIME} + \text{I}(\text{TIME}^2)$

Value Std.Error DF t-value p-value

(Intercept) 1.1851812 0.17176406 13 6.900054 0.0000

TIME 0.0058821 0.00239597 13 2.454976 0.0289

I(TIME^2) -0.0000094 0.00000646 13 -1.458151 0.1685

Correlation:

(Intr) TIME

TIME -0.759

I(TIME^2) 0.616 -0.969

Standardized Within-Group Residuals:

Min Q1 Med Q3 Max

-2.6101900 -0.5977584 0.1964076 0.5137681 1.5550877

Number of Observations: 23

Number of Groups: 8

```
> lme_MED<-lme(log(MICRO+1)~TIME+I(TIME^2), na.action=na.omit, data=MED,  
method="ML", random =~1|TK)
```

```
> summary(lme_MED)
```

Linear mixed-effects model fit by maximum likelihood

Data: MED

AIC BIC logLik
20.08512 25.76259 -5.042558

Random effects:

Formula: ~1 | TK

(Intercept) Residual

StdDev: 3.837031e-06 0.3012855

Fixed effects: $\log(\text{MICRO} + 1) \sim \text{TIME} + \text{I}(\text{TIME}^2)$

Value Std.Error DF t-value p-value

(Intercept) 1.6257819 0.14683385 13 11.072256 0.0000


```
TIME      0.0059020 0.00221990 13 2.658674 0.0197
I(TIME^2) -0.0000105 0.00000606 13 -1.736812 0.1060
```

Correlation:

(Intr) TIME

```
TIME      -0.743
```

```
I(TIME^2) 0.609 -0.972
```

Standardized Within-Group Residuals:

```
      Min      Q1      Med      Q3      Max
-1.9229672 -0.8555631 0.0888646 0.6679971 1.9073278
```

Number of Observations: 23

Number of Groups: 8

```
> lme_HIGH<-lme(log(MICRO+1)~TIME+I(TIME^2), na.action=na.omit, data=HIGH,
method="ML", random =~1|TK)
```

```
> summary(lme_HIGH)
```

Linear mixed-effects model fit by maximum likelihood

Data: HIGH

```
      AIC      BIC  logLik
9.553954 15.44422 0.2230229
```

Random effects:

Formula: ~1 | TK

(Intercept) Residual

```
StdDev: 0.1239541 0.2133538
```

Fixed effects: log(MICRO + 1) ~ TIME + I(TIME^2)

```
      Value Std.Error DF  t-value p-value
(Intercept) 2.0016399 0.11329758 14 17.667101 0e+00
TIME         0.0071754 0.00150005 14 4.783461 3e-04
I(TIME^2)    -0.0000175 0.00000409 14 -4.294513 7e-04
```

Correlation:

(Intr) TIME

```
TIME      -0.683
```

```
I(TIME^2) 0.555 -0.970
```

Standardized Within-Group Residuals:

```
      Min      Q1      Med      Q3      Max
-1.80206196 -0.79953521 -0.02417999 0.90814375 1.43661016
```

Number of Observations: 24

Number of Groups: 8

```

> shapiro.test(TRICLO$D_SVL)

      Shapiro-Wilk normality test

data:  TRICLO$D_SVL
W = 0.9851, p-value = 0.2118

> SVL.aov<-aov(D_SVL ~ TTT, data=TRICLO)
> summary(SVL.aov)
      Df Sum Sq Mean Sq F value Pr(>F)
TTT      4  0.0403  0.0101  0.1105 0.9786
Residuals 115 10.4915  0.0912

> LATE<- subset(TRICLO, subset=TIME=="336")

> TL.aov<-aov(D_TL ~ TTT, data=TRICLO)
> summary(TL.aov)
      Df Sum Sq Mean Sq F value Pr(>F)
TTT      4  0.878  0.219  0.3054 0.8739
Residuals 115 82.637  0.719

> LATE.aov<-aov(D_TL ~ TTT, data=LATE)
> summary(LATE.aov)
      Df Sum Sq Mean Sq F value Pr(>F)
TTT      4  1.2371  0.3093  0.3483 0.8434
Residuals 35 31.0757  0.8879

```