Developing a Bioassay for Triclosan Toxicity

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Abstract

The use of triclosan, 5-chloro-2-(2,4-dichlorophenoxy) phenol or TCS, began with soaps and has extended to products all throughout the household. This chemical is now one of the most commonly detected organic compound in surface waters. It has been proven to accumulate in tissue, cause toxicity to aquatic organisms, and impair muscle contraction. It is critical to develop a sensitive and economic bioassay for a chemical so prevalent in our environment. This study sought to determine the affects of TCS on embryonic development, as well as the biological system that would detect the compound at levels found in surface waters. The Microtox assay performed revealed that TCS was highly toxic to *V. fischeri*. Zebrafish, *Danio rerio,* toxicity assay confirmed TCS to cause deformities in the spine. TCS was found to increase the expression of ndr2 and pkd2, two genes that are known to be important for spinal cord formation. Furthermore, actin2b expression was severely decreased in TCS treated embryos compared to control embryos. Embryonic defects were also observed in chick embryos with embryos treated with TCS having smaller body size and irregular eye pigmentation compared to control embryos of the same stage. Thus, our study suggests potential use of expression of spinal cord genes in zebrafish and chick embryos as good bioassays for TCS toxicity in eukaryotes.

Introduction

Triclosan, 5-chloro-2-(2,4-dichlorophenoxy) phenol or TCS, is an antibacterial compound found in personal care products such as toothpaste, shampoos, cosmetics, household cleaning products, and preservatives. Its antibacterial characteristic lies in its ability to inhibit the synthesis of fatty acids, a critical component in cell membranes and metabolism (Gaume et al., 2012). It was introduced in 1968 and is increasingly being used in various other products(Oliveira, et al., 2009). Regulators have deemed TCS safe in current quantities used in products. However, it is now one of the most commonly detected organic products found in wastewater (Gaume et al., 2012). TCS levels detected in the USA were 13,700 - 86,200 ng/L (Bedoux et al., 2012). The bioaccumulation of this compound may have adverse effects beyond aquatic life. TCS is relatively hydrophobic, moderately soluble in water, and may take days to degrade. In addition, TCS reacts to ozone and chlorine to produce dioxins, known to be endocrine disruptors, and other toxic by-products (Bedoux et al., 2012, Farre et al., 2007). Although TCS concentrations are relatively low, studies have shown bioaccumulation in fish tissue and filamentous algae at 100-150 µg/kg (Gaume et al., 2012). Accumulation in such primary food source could lead to biomagnification of TCS in the food chain, ultimately being toxic not only to aquatic life but having adverse effects on a larger scale.

Previous studies on TCS have shown the compound to be lethal to fishes at high concentrations while effecting development at lower concentrations. One study proves concentrations 0.7 - 0.9 mg/l to be lethal and 0.3 - 0.5 mg/l delay growth (Oliveira et al., 2009). Furthermore, there are rising concerns about the effects of TCS on non-aquatic organisms, including humans. TCS is suspected of interfering with the endocrine system and hormone signaling (Bedoux et al., 2012). Such a disruption would lead to effects on embryonic development and later growth, as well as, fertility (Farre et al., 2007). TCS has been proven to impair cardiac and skeletal muscle function by altering the activity of type 1 ryanodine receptor, a calcium channel in the cell membranes. Rats injected with 25 mg/kg TCS suffered 25.3 ± 15.7% decrease in cardiac output while fathead minnows showed reduced swimming performance (Cherednichenko et. al,

2012). Although it is proven that TCS causes toxicity in aquatic life and certain non-aquatic organisms, the effects of TCS at the genetic level, as well as on varying tissues and organs, are unknown. It is necessary, then, to develop a sensitive mechanism to screen for such a compound that is prevalent in our environment.

The aim of this study is to develop an easier and more sensitive bioassay for TCS that would include effects on non-aquatic life and could be routinely used in any lab. In addition, we plan to identify genetic biomarkers within the assay to investigate the mechanism by which TCS causes toxicity.

Materials and Methods

Vibrio fischeri and Microtox solutions were purchased from Modern Water (New Castle, DE, USA). Zebrafishes were obtained from Carolina Bio Supplies (Burlington, NC,USA). Tanks and equipment to maintain fish environment was purchased from a local pet store. miRNeasy mini kit, high capacity RNA-to-cDNA kit, and gene expression assays were ordered from Invitrogen (Carlsbad, CA, USA). Two dozen fertilized eggs were purchased from the local grocery store. Irgasen (triclosan) was obtained from Sigma Aldrich (St. Louis, MO, USA). Triclosan stock solution was prepared with acetone to a concentration of 9 mg/ml.

Microtox Assay

The Microtox is a common assay used to assess the toxicity and ecological risk of various chemical compounds. This assay follows the protocol established by Azure Environmental and matches EPA screening procedure. The freeze-dried bioluminescent bacteria was mixed with 1.0 ml of reconstituting reagent and aerated several times to prepare for the toxin. Five hundred micro-liters of the reagent mixture was pipetted into sample cuvets in B1 to B5 in the Microtox Model 500 Analyzer. The luminescence of the *V. fischeri* is read and recorded after 15 minutes. TCS solution of 0.9 µg/L concentration was added to 250 ul of osmotic adjustment solution in cuvet A5 to prepare the toxin

solution. One milliliter of the toxin solution was mixed with 1 ml of diluent in cuvet A4 and aerated several times. Similarly, 1 ml of the toxin solution in A4 was pipetted into 1 ml of diluent in A3 and aerated. This serial dilution was repeated to cuvets A2 and A1. The diluted toxin solutions were used to treat *V. fischeri* and the luminescence is read and recorded after 5 minutes. This procedure was repeated with a phenol solution of 94.5 mg/L concentration to be used as a standard.

Collecting Zebrafish embryos

Male and female zebrafishes were obtained from Carolina Bio Supplies. The fishes were kept in tanks at 25^oC on 14/10 light cycle and were fed once daily. The water was cleaned and syphoned daily to prevent waste buildup. A mating cage, a pyrex container with netting, was put in late in the day. The embryos were collected the following morning and rinsed with Hank's embryo media. The media is composed of 1.0 ml stock 1 (8.0g KCl to 100ml), 0.1 ml stock 2 (0.67g $Na_2HPO_4*7H_2O$ and 0.60g KH_2PO_4 to 100ml), 1.0 ml stock 3 (1.92g $CaCl_2*2H_2O$ to 100 ml), stock 4 (2.46g $MgSO_4*7H_2O$ to 100ml), 0.35g $NaHCO_3$, and 96.5 ml H_2O.

Zebrafish Embryo Toxicity Assay

Embryos collected were divided into six wells so that two wells could be prepared for each of the three conditions. Two wells contained triclosan stock of 9 mg/ml diluted to 0.9 µg/L in Hank's embryo media, two with 0.9 µg/L acetone in Hank's embryo media, and two with Hank's embryo media. The plate was kept in an incubator at 28C and examined after 12, 24, 48, and 96 hours by phase contrast using a Nikon microscope.

Gene expression assay

Embryos collected were placed in Hank's embryo media and divided into two wells. Triclosan stock of 9 mg/ml was diluted into one well for a final concentration of 0.9 µg/L. The wells were kept in an incubator at 28ºC for 24 hours. The media was then removed and the embryos snapfrozen with liquid nitrogen for later RNA purification.

The embryos' RNA was purified using miRNeasy Mini Kit following the Qiagen quick-start protocol (Carlsbad,CA, USA). The RNA was then converted to cDNA using the High Capacity RNA-to-cDNA kit from Applied Biosystems (Carlsbad, CA, USA). The DNA obtained was diluted to 100 ng/µl of cDNA per 20 uL and used in GAPDH, pkd2, actinb2, ndr2, and gli2a gene expression assays (Carlsbad, CA, USA). The genes ndr2, gli2a, and pkd2 have been identified with essential function in spinal cord development (Haffter et al, 1996). The genes GAPDH and actinb2 are reference genes.

Incubating Chick eggs

The eggs were placed in an incubator set to 38ºC. Eight eggs were injected with 30 ul of 0.9 mg/ml TCS in water, eight eggs with 30 µl of 0.9 mg/ml acetone in water, and seven with 30 ul of water. The point of injection is covered with tape to prevent from desiccation. The eggs were kept in the incubator for 7 days.

Maintaining neurons

Neurons were plated into six wells. Media used in neuron cultures contain 100µg/ml NGF, insulin, selenium, transferrin, and 0.5 mg/ml BSA. Two wells were treated with 9 mg/L TCS in media , two with 9 mg/L acetone in media, and two with only media. The wells were kept in CO_2 in a desiccator at 37ºC to maintain appropriate pH.

Results

Microtox Assay

Previous studies have shown that TCS can be detected by the Microtox assay at levels found in the environment (Farre et al., 2007). The EC50 value, the concentration at which half the bacteria were killed, of TCS in one study was 0.28 µg/ml with the lowest observed effect concentration at 0.10 µg/ml (Farre et al., 2007). To determine whether the TCS ordered would behave as expected, we performed the Microtox assay. The EC50 value obtained from the luminescence recorded after 5 minutes of exposure to the TCS toxin is 0.134 mg/L (Fig. 1). The difference in the EC50 values for TCS may be reflective of the source of the compound. The study isolated the compound from ground and surface waters, while the TCS used in this study was purchased from Sigma Aldrich (St. Louis, MO,USA). The standard, phenol, had an EC50 value of 23.157 mg/L. The immense difference in the EC50 values indicates that TCS is highly toxic to the bacteria. Although this assay was able to detect TCS at low levels, it only confirms the efficiency of TCS as an antimicrobial but does not reveal its toxicity to eukaryotes.
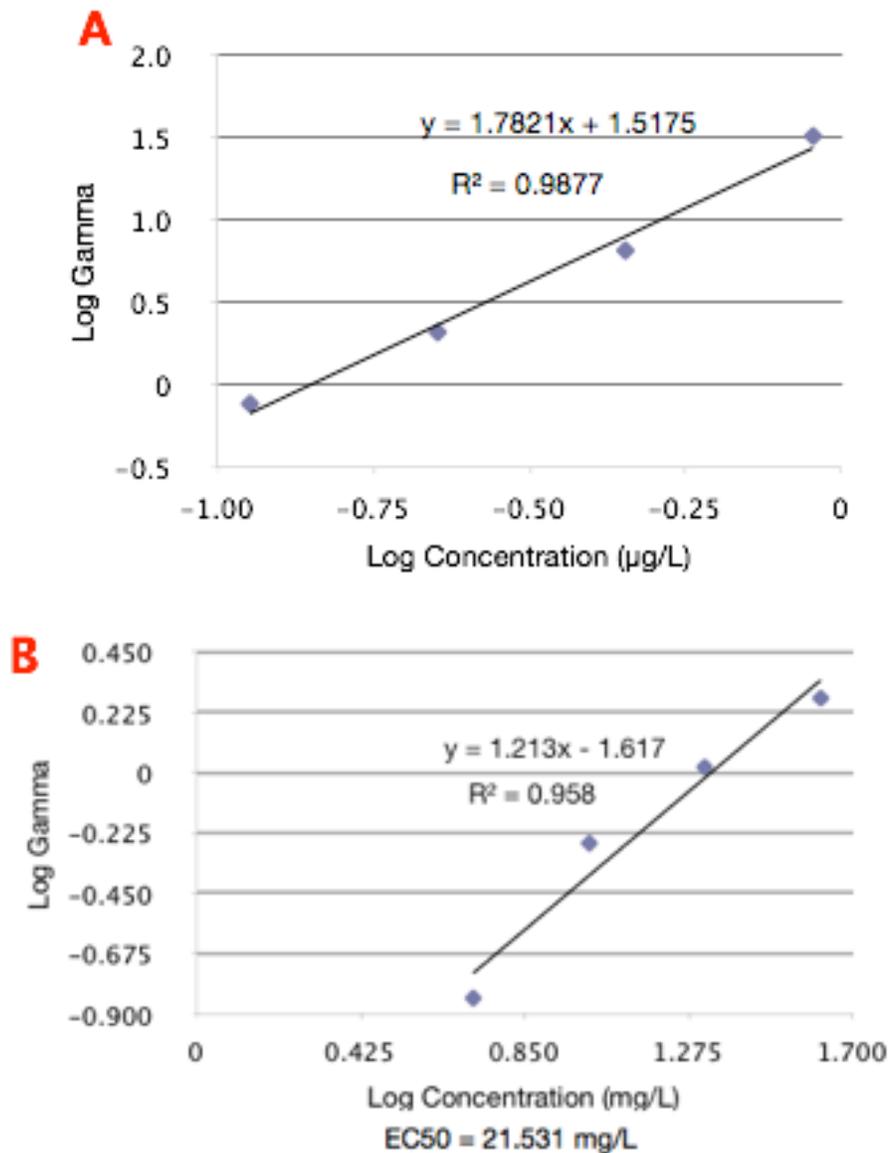
Figure 1. These graphs show the decreasing rate of bioluminescence of *V. fischeri* in relation to decreasing concentration of toxin. **A** Triclosan Microtox Assay. *V. fischeri* were treated with TCS concentrations of 0.9, 0.45, 0.225, and 0.1125 µg/L. **B** Phenol Microtox Assay. *V. fischeri* were treated with phenol concentrations of 94.50, 47.25, 23.62, 11.81 mg/L. The slope and intercept obtained from each graph was used to calculate the EC50 value of the toxin.

TCS causes spinal deformities in zebrafish

Though TCS was found to be an effective antimicrobial agent, the Microtox does not show toxicity to eukaryotic systems. Previous studies have shown zebrafish to be a good model to study TCS toxicity (Olivier et al., 2009). Zebrafish embryos exposed to TCS developed showed abnormal growth. After 96 hours, there was higher mortality in TCS than in the two control conditions. Some of the embryos that have developed possessed a curled up tail while others had a curled down tail. While in the controls, the zebrafishes developed a straight tail without any bending of the spine (Fig. 2B). One zebrafish in TCS showed a severe kink in the spine above vital organs that left the body of the fish bent at an angle (Fig. 2D). However prior to 96 hours of exposure to TCS, the embryos appeared to develop just as those in control and solvent control (Fig. 2A, Fig. 2C). The embryos showed normal development of head, eyes, and spine. No deformities were detected at this time.
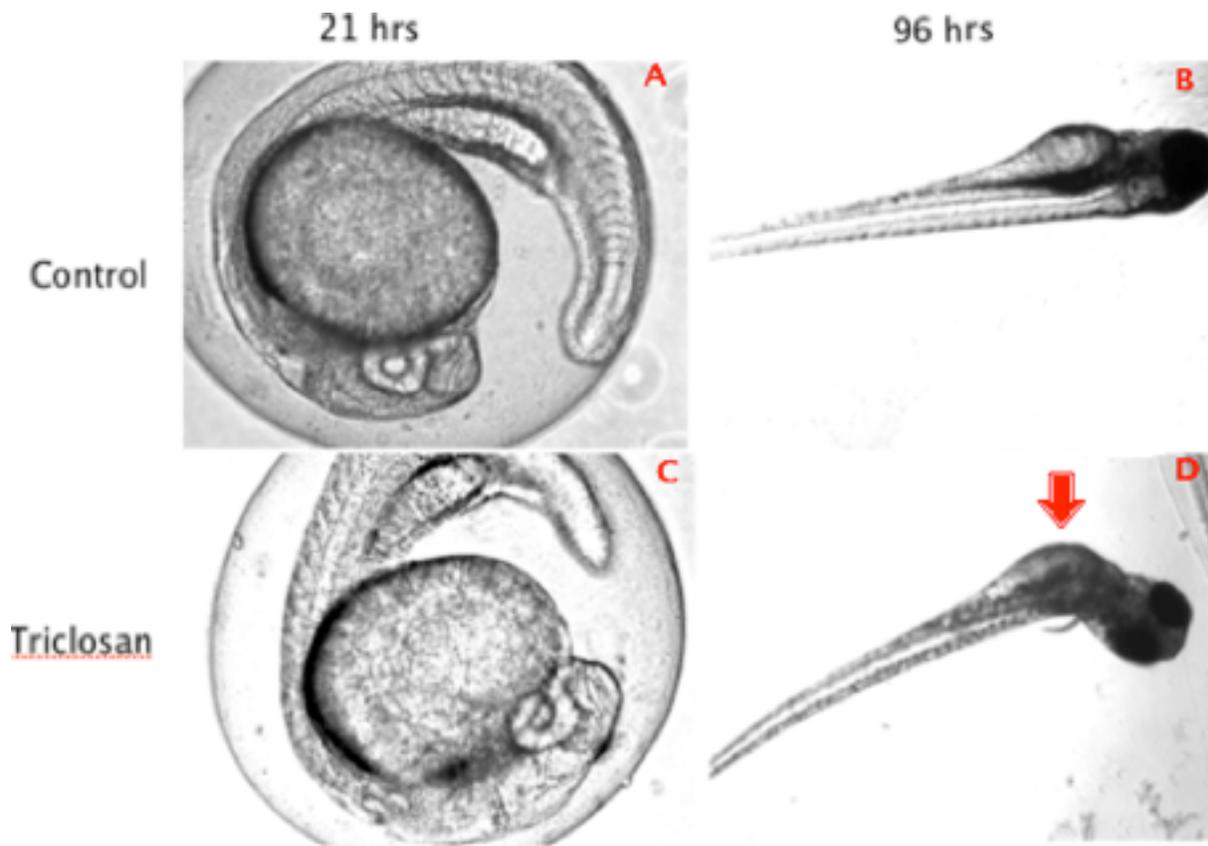
Figure 2. Exposure of Zebrafish embryos to TCS. **A** normal embryonic development after 21 hrs **B** normal embryonic development after 96 hrs **C** embryo exposed to 0.9 mg/L TCS after 21 hrs **D** embryo exposed to 0.9 µg/L after 96 hrs developed a kinked spine.

TCS Affects Gene Expression

An occurrence of a phenotype at 96 hrs suggests that there must be changes in genes regulating spinal cord development at an earlier time point. Therefore, examining gene expression may provide an earlier and more sensitive detection method for TCS toxicity. Previous studies have identified number of genes as being essential for spinal cord development (Hafter et. al, 1996). Three of these genes - *cyc (also known as ndr2), yot (also known as gli2a), and dtr (also known as pkd2)* had either early roles in

spinal cord formation or showed similar mutant phenotypes as TCS treated embryos. Ndr2, nodal-related 2, is linked to the expression of anterior axial tissues involved in the formation of prechordal plate and notochord (Rebagliati et al,. 1998). Gli2a is involved in the development of lateral mesoderm and works with other Glis in the expression of the neural tube (Ke et al., 2008). Pkd2 is associated with the expression of the lateral plate mesoderm (LPM) and left-right patterning (Schottenfeld et al., 2007). To determine the changes in expression of these three genes, qPCR analysis for cyc, yot and pkd2 were performed on RNA from embryos treated with control, solvent control and TCS treated embryos. The gene expression of actin and GAPDH were used as controls to normalize the levels of the target genes. The expression of gli2a did not change with exposure to TCS (Fig. 3). In contrast, there was a slight increase in ndr2 and a two-fold increase in pkd2 in embryos exposed to TCS. Though there was a change in the gene expression of the target genes, the control genes did not behave as expected under the two conditions. GAPDH was not detected by the PCR. Actin, on the other hand, severely decreased with exposure to TCS (Fig. 3).
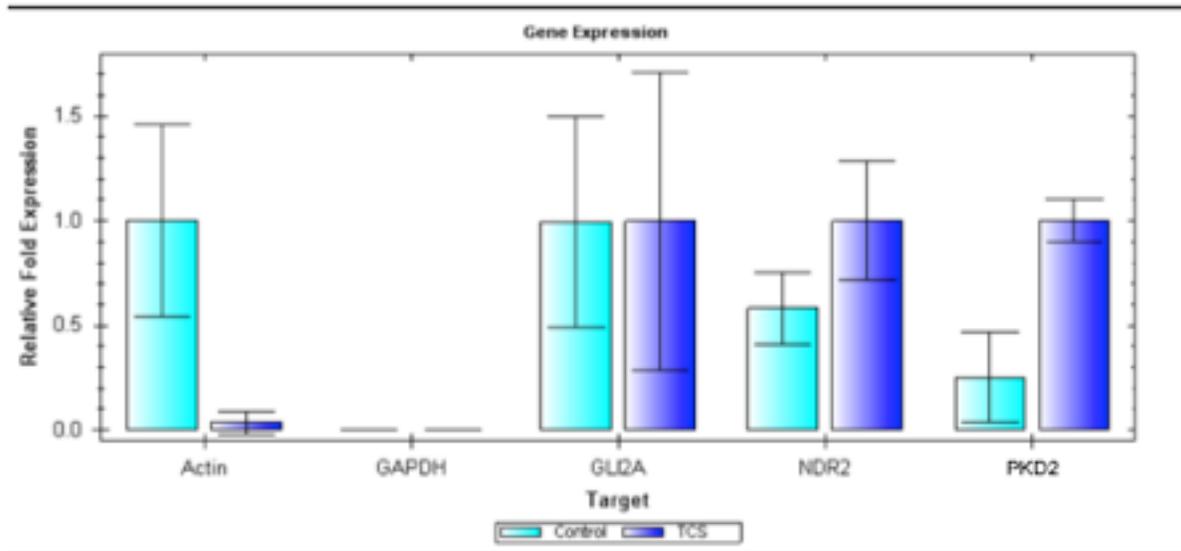
Figure 3. The q-PCR gene expression of gli2a, ndr2, pkd2, GAPDH, and actin2b in control and TCS treated zebrafish embryos.

Exposure to TCS causes stunted growth in Chick Embryos

To determine if TCS causes similar spinal malformations in other organisms, chick embryos were injected with TCS. All eight control eggs developed into embryos. In comparison, only 37.5% of eggs developed in solvent control Figure 5. However, those embryos that developed were comparable to those of control. There was also a low percentage of embryos formed in TCS treated eggs. Fifty percent of TCS treated eggs developed and 50% of the developed showed abnormalities. In contrast to the solvent control eggs, TCS treated embryos showed stunted growth and irregular eye pigmentation (Figure 6 B). Embryos treated with TCS were much smaller in length when compared to embryos of a similar stage. Two of the four embryos had no distinguishable limbs and eyes.
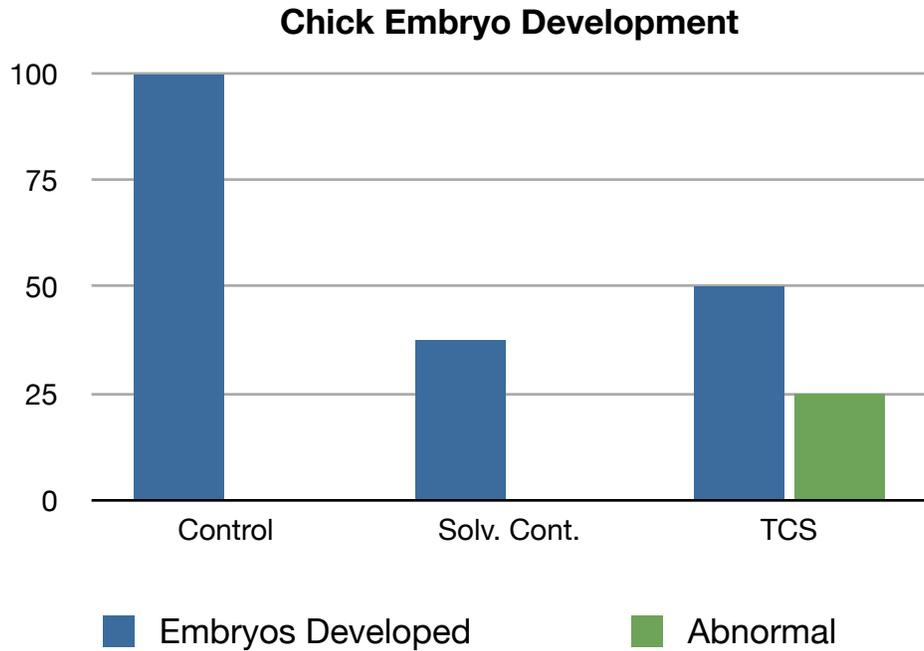
Chick Embryo Development

Figure 5. This graph details the percentage of embryos developed, the number of normal embryos, and abnormal embryos in each condition.
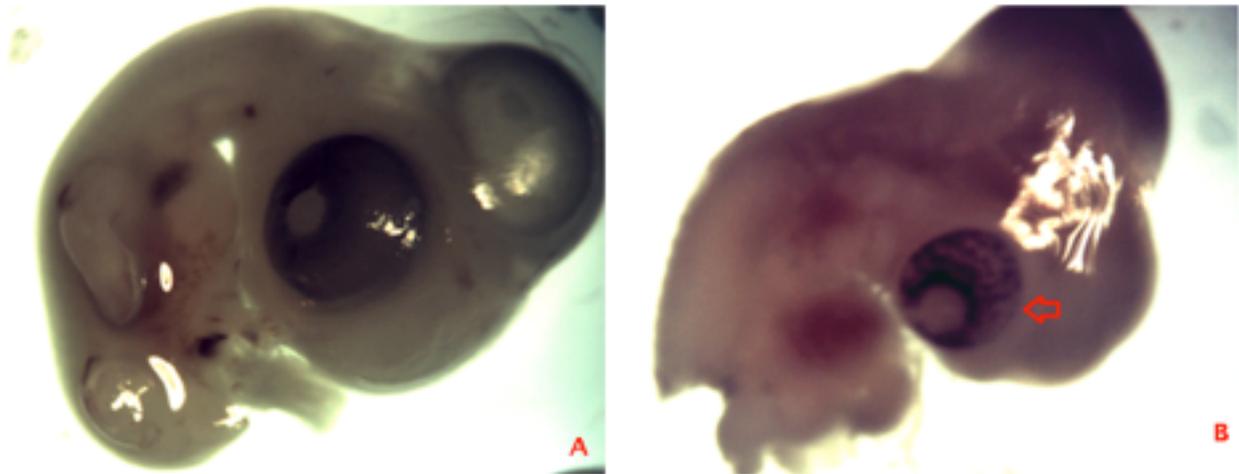

Figure 6. Chick Toxicity Assay **A** control embryo approximately at stage 26-27. The length of the body is 1.4 cm and the length of the head is 0.8 cm. **B** TCS - treated embryo at approximately stage 25. The length of the body is 0.8 cm and the length of

the head is 0.5 cm. (The glow at the head of the embryo is a glare from the microscope.)

High Concentrations of TCS causes neuronal death

The defects in spinal cord structure may be due to aberrant patterning of spinal neurons or spinal cord neuronal death. Therefore to examine the effects at the cellular level, neurons were treated with TCS to look at viability and neurite outgrowth. Neurons treated with TCS concentrations of 3, 0.9, 0.6, 0.09, 0.03, and 0.009 µg/L did not shown changes in viability and neurite outgrowth (data not shown). However, there were neuronal effects observed when the neurons were treated with a higher concentration of TCS In comparison to control neurons in media, neurons treated with 9 µg/L TCS showed severe damage to neuronal processes (Fig. 4B). The axons were fractured rather than smooth like the axons of the control neurons (Fig. 4 A). Axons allow neurons to communicate and send signals to the brain. When the axons begin to fracture, the neurons cannot function properly and inhibit motor and sensory signaling. This suggests that although TCS does not cause neuronal death at low concentrations, TCS has the capacity to be a neurotoxin at high concentrations.
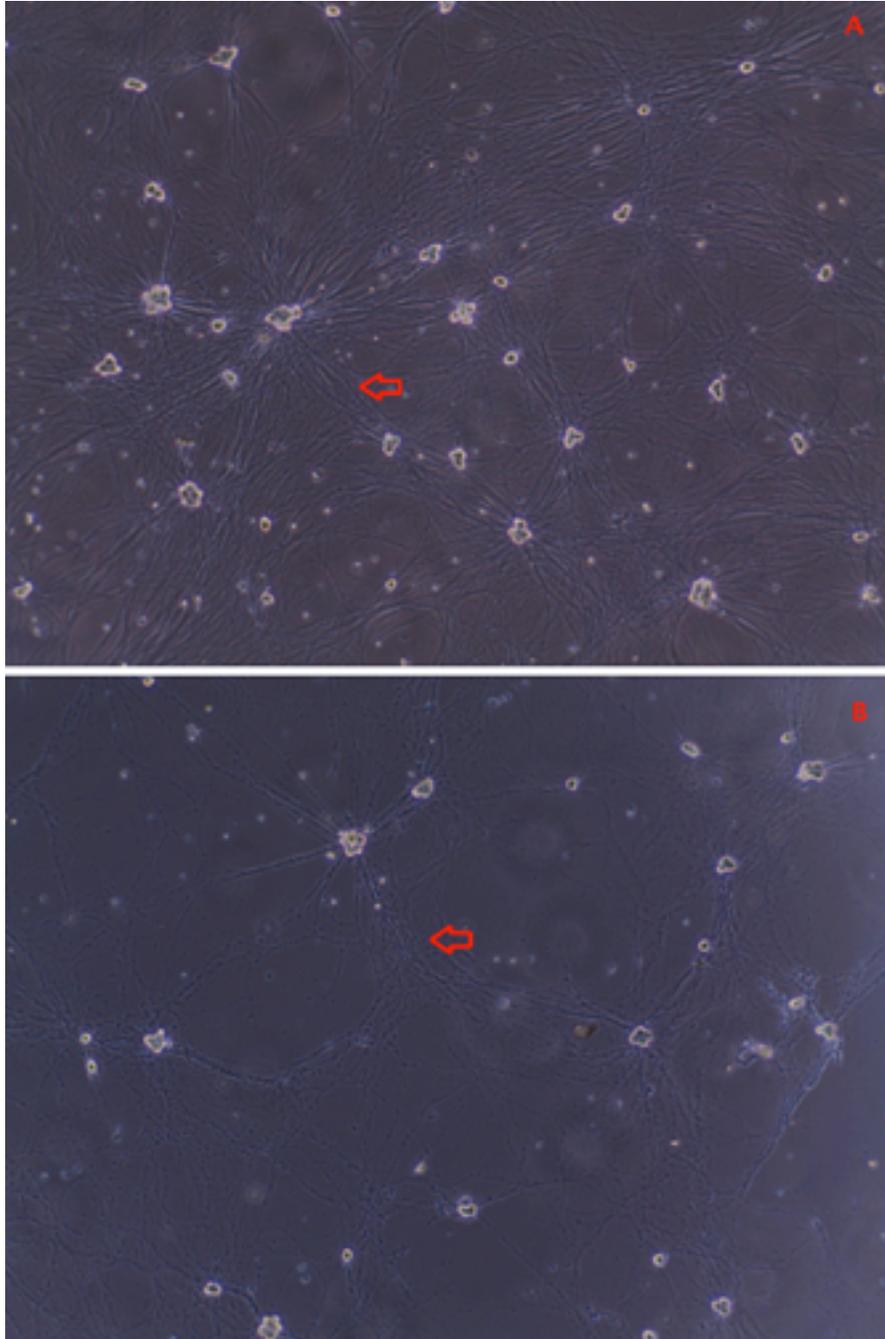
Figure 4. Neurons taken by a phase contrast microscope. **A** neurons in the control medium C2 **B** neurons cultured in 9.0 mg/L TCS in C2 began to bleb and axons were breaking apart. Arrow indicates the blebs on the axons.

Discussion

In this study we have shown the toxicity of TCS on zebrafish and chick embryos. While embryonic development is similar across various eukaryotic organisms, the effects of TCS varies between zebrafish and chick. Zebrafish embryos exposed to TCS developed curled spines and, in one particular case, a kinked angular spine. In addition, there was a higher mortality rate in embryos exposed to TCS than in control embryos. This is in agreement with findings of Oliveira et al (). On the other hand, chick embryos exposed to TCS showed significantly stunt growth and irregular eye pigmentation. It was unclear if there were changes in the spine formation in chick embryos. In addition, TCS reacts with ozone and chlorine to form dioxins that are endocrine disruptors. Interestingly, a recent study detected similar spine abnormalities in fathead minnows following Bisphenol exposure due to effects as an endocrine disruptor (Warner et al., 2007). The similarity in the toxicity of TCS and bisphenol suggests that some of the affects observed may be due to metabolites rather than TCS itself.

The defects in embryonic development in zebrafish were associated with changes in gene expression after 24 hours of TCS exposure. Our results showed that there was an increase in the gene expression of two genes : ndr2 and pkd2 in TCS treated embryos compared to control embryos, with gli2a being unaffected. However, the control genes did not behave as expected. As a result, the data could not be normalized and validated. Though the reference genes were not effective controls, the expression of two of the target genes increased with exposure to TCS while the expression of actin2b, the reference gene, dramatically decreased. This suggests that differences in gene expression of ndr2 and pkd2 between control and TCS treated embryos was not due to differential amounts of the RNA being used for PCR. In order to fully understand the genetic changes, the experiments needs to be repeated with other control genes. It would also be interesting to determine if the expression of the homologs of these genes is altered in chick embryos.

An interesting finding of our study was a severe decrease of actin2b in TCS treated embryos that was consistently observed in multiple experiments. Actin functions in muscle contraction, as well as, the cell cycle and division. Its abnormal expression

could impair the function of muscles and result in the lock of muscles on one side. The pull of such a deformity would then cause the spine to curl. It is therefore possible that the defects in the spinal cord curvature observed in zebrafish and fathead minnows is more a result of musculature defects rather than neuronal defects. This is consistent with our observations that there was not changes in neuronal health upon treatment with lower concentrations of TCS. The effects of TCS on actin may explain the impairment of cardiac and skeletal muscle observed by Cherednichenko et al (). Furthermore, the decrease of actin can also affect cell growth. Actin plays a critical role in the cytoskeleton and cell division. Actin's interaction with myosin allows the microtubules to contract during cytokinesis. The inhibition of such a process may prevent cells from complete separation after division and ultimately affect the growth of tissues and organs as well as the viability of individual cells during development. Thus, the role of actin in various processes may bridge the variation of toxicity between the zebrafish and chick embryos and needs to be further explored to fully understand the toxic effects of TCS.

We have shown that exposure to TCS caused spinal abnormalities in zebrafish embryos and stunted growth in chick embryos. A qPCR of glia2a, pkd2, and ndr2 genes in zebrafish embryos showed that TCS caused a increased expression of pkd2 and ndr2 while gli2a remained the same. Interestingly, TCS caused a dramatic decrease in the expression of actin2b. Though neurons showed toxicity when treated with a high concentration of TCS, neurons were unaffected by low concentrations.

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