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# Toxicological Effects of Antimicrobials (Triclosan and Triclocarban) and Cyanopeptides (Anabaenopeptins and Cyanopeptolins) in the Nematode C. Elegans

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by

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ABSTRACT

TOXICOLOGICAL EFFECTS OF ANTIMICROBIALS (TRICLOSAN AND TRICLOCARBAN) AND CYANOPEPTIDES (ANABAENOPEPTINS AND CYANOPEPTOLINS) IN THE NEMATODE *C. ELEGANS*

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Environmental contaminants are increasingly detected in surface water, soil, and sediment. There is a concern that environmental contaminants, such as cyanpeptides and antimicrobials, pose potential harm to environmental and public health. It is important that scientists evaluate the potential impacts that cyanopeptides and antimicrobials may have on the environment and public health in order to guide stakeholders in determining appropriate policies and regulations. Here we investigated the toxicological effects of two types of commonly detected environmental contaminants, antimicrobials (triclosan and triclocarban) and cyanopeptides (anabaenopeptins and cyanopeptolins), using the model organism *Caenorhabditis elegans* (*C. elegans*). Chapter 1 introduces the model organism *C. elegans* and proposes the use of this nematode model for environmental toxicology studies. Acute lethality and sublethal toxicity of cyanopeptides (anabaenopeptins and cyanopeptolins) and antimicrobials (triclosan and triclocarban) are identified using *C. elegans* in Chapters 2 and 3. The examined toxicity endpoints included reproduction, hatching time, growth rate, lifespan, age-related vulval integrity, germline toxicity, and stress response. In Chapter 4, we examined the potential mechanism of toxicity of the antimicrobials, triclosan and triclocarban. In Chapter 5, we identify the toxicological effects of degradation products produced by triclosan and triclocarban after these chemicals enter the environment. The research presented here demonstrates that cyanopeptides (anabaenoptpins and cyanopeptolins) and antimicrobials (triclosan and triclocarban) pose potential harm to the environment

and establishes endpoints for future toxicological studies in the model organism *C. elegans*. These findings will provide insight into the toxicological effects and mechanism(s) of toxicity that will add to our knowledge of the effects of these environmental contaminants, as well as provide scientific evidence to policy-makers and regulatory agencies for the creation and amendment of environmental regulation policies.

Dedication

In memory of my mentor and friend,

Ben Barres

(1954-2017)

Ben entered my life during a pivotal time in the fall of 2015. Ben guided me through this difficult time in my life with grace, humor, and empathy. In our short time together, Ben taught me many lessons that I will carry with me for the rest of my life. Thank you, Ben, for inspiring and supporting the next generation of scientists. Thank you, Ben, for making my life possible as a transgender scientist. Thank you, Ben, for teaching me what it means to live a life with grace, resiliency, empathy, character, and integrity. I promise to make you proud.

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LIST OF ABBREVIATIONS

| | |
|---|---|
| °C | Degree Celsius |
| µg/L | Micrograms per liter |
| 2,8-DCDD | 2,8-dibenzodichloro-p-dioxin |
| *act-1* | Actin-1 |
| *age-1* | Phosphoinositide 3-kinase |
| AHP | 3-amino-6-hydroxy-2-piperidone |
| *akt-1/2* | Protein kinase B |
| AP | Anabaenopetin |
| Avid | Age-related vulval integrity defect |
| BPA | Bisphenol A |
| BαP | Benzo-α-pyrene |
| *C. elegans* | Caenorhabditis elegans |
| cDNA | Complimentary DNA |
| *clec-60* | C-type LECtin |
| CYP | Cyanopeptolin |
| *daf-12* | Nuclear hormone receptor |
| *daf-15* | Raptor homolog |
| *daf-16* | Forkhead-box Class O |
| *daf-18* | Phosphatase and tensin homolog |
| *daf-2* | Insulin-like growth factor receptor |
| DCC | Dichlorocarbanilide |
| DEB | Dynamic energy budgets |
| DMSO | Dimethyl sulfoxide |
| DOM | Dissolved Organic Matter |
| *E. coli* | Escherichia coli |
| EB | Evans blue |
| ECHA | European Chemicals Agency |
| EDCs | Endocrine-disrupting compounds |

| | |
|---|---|
| EFSA | European Food Safety Administration |
| EPA | Environmental Protection Agency |
| FDA | Food and Drug Administration |
| FOXO | Forkhead-box Class O |
| FUdR | 5-fluoro 2-deoxyuridine |
| g | Gram |
| GFP | Green fluorescent promoter |
| GH | Growth hormone |
| GHS | Globally Harmonized System |
| h | Hour |
| $H_2O$ | Water |
| IGF | Insulin-like growth factor |
| *ist-1* | Insulin receptor substrate homolog |
| KCl | Potassium chloride |
| $KH_2PO_4$ | Monopotassium phosphate |
| L1-L4 | Larval stages 1-4 |
| $LC_{50}$ | Lethal concentration |
| *let-363* | Target of rapamycin homolog |
| *lin-42* | Period protein homolog |
| $LRP_2$ | Lipoprotein-related protein 2 |
| *lys-7* | Lysozyme-like protein 7 |
| M | Molar |
| MC | Microcystin |
| MeOH | Methanol |
| Mg | Milligram |
| mg/kg | Milligram per kilogram |
| mg/L | Milligrams per liter |
| $MgSO_4$ | Magnesium sulfate |
| mM | Millimolar |

| | |
|---|---|
| $Na2HPO_4$ | Disodium phosphate |
| NaCl | Sodium chloride |
| NaClO | Sodium hypochlorite |
| NaOH | Sodium hyroxide |
| ng | Nanogram |
| NGM | Nematode growth media |
| PBDEs | Polybrominated diphenyl ethers |
| PCBs | Polychlorinated biphenyls |
| PCDDs | Polychlorodibenzo-p-dioxins |
| *pdk-1* | Phosphoinositide-dependent protein kinase-1 |
| *pgp-5* | P-GlycoProtein |
| *pip2* | Phosphoinositide-3,4-P2 |
| *pip3* | Phosphoinositide-3,4,5-P3 |
| qPCR | Quantitative polymerase chain reaction |
| RNAi | RNA interference |
| *rsks-1* | Ribosomal protein S6 kinase beta |
| *sgk-1* | Serum/Glucocorticoid Regulated Kinase 1 |
| *skn-1* | Protein skinhead-1 |
| TCC | Triclocarban |
| TCS | Triclosan |
| TGF-B | Transforming growth factor beta |
| TKTD | Toxicokinetic-toxicodynamic |
| TOR | Target of rapamycin |
| UV | Ultraviolet |
| *vit* | Vitellogenesis |
| WHO | World Health Organization |
| ΔΔCt method | Delta delta Ct method |
| µg/kg | Microgram per kilogram |
| µL | Microliter |

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"No one is born hating another person because of the color of his skin,
or his background, or his religion. People must learn to hate,
and if they can learn to hate, they can be taught to love, for love
comes more naturally to the human heart than its opposite."

-Nelson Madela, "Long Walk to Freedom"

CHAPTER 1:

General Introduction

***Introduction***

The ongoing development of science and technology creates a continuous synthesizing of new chemicals used for industrial production and human life (NRC, 2015; Kovac, 2015). Assessment of these new chemicals and environmental contaminants for potential toxicity is an urgent and ongoing issue (Gao et al., 2015). In recent years, much attention has been focused on environmental contaminants, such as cyanopeptides and antimicrobials, as there has been a call for regulations on the use and detection of chemicals and environmental contaminants (FDA, 2016). Given the need for information regarding the toxicological effects of cyanopeptides and antimicrobials, we examined the toxicological effects of these environmental contaminants, cyanopeptides and antimicrobials, using the invertebrate model *Caenorhabditis elegans* (*C. elegans*).

This study examines the toxicological effects of antimicrobials (triclosan and triclocarban) and cyanopeptides (anabaenopeptins and cyanopeptolins) in the model organism *C. elegans*. First, we established defined endpoints to study the toxicological effects of antimicrobials (triclosan and triclocarban) and cyanopeptides (anabaenopeptins and cyanopeptolins) (Ch. 2 and 3). The examined toxicity endpoints included reproduction, hatching time, growth rate, lifespan, age-related vulval integrity, germline toxicity, and stress response. Second, we studied the mechanism of toxicity of the antimicrobials, triclosan (TCS) and triclocarban (TCC), using genetic analysis with a series of worm mutants and gene expression in wildtype worms (Ch. 4). This study assessed the role of the insulin-like growth factor pathway, an important pathway involved in development and longevity, in the TCS- and TCC-induced toxicity. Third, we identified the toxicological effects of TCS and TCC degradation products (Ch. 5). This study employed the methods established in the previous chapters to examine the toxicological effects of the degradation products of TCS and TCC compared to the parent compounds. The proposed research examined the toxicological effects of widely detected environmental contaminants, cyanopeptides and antimicrobials, and established endpoints for future toxicological studies in the invertebrate model *C. elegans*. These findings provide insight into the toxicological effects and mechanism(s) of toxicity that will add to our knowledge of the effects these environmental contaminants have on the environment and human health. Furthermore, better understanding of the connection between contaminants and diseases can help inform future environmental regulation policies.

***Background***

Scientists have gained extensive knowledge in biology and toxicology using *in vivo* and *in vitro* models (Barre-Sinoussi and Montagutelli, 2015). The expectation of toxicity testing is that the information obtained from a specific animal model will apply to a variety of biological systems (Krewaski et al., 2010). Traditional toxicology used mammalian models as the "gold standard" because of the similar development processes, organ systems, and signaling pathways of those found in humans. However, mammalian models are not perfect, as human trials sometimes fail to predict outcomes in the general population (Hunt, 2017).

Drawbacks of using mammalian and fish models are that these models are expensive and time-consuming to perform (Planchart et al., 2017). Additionally, the use of only one rodent model has been shown to predict specific toxicity in humans only 50% of the time (Hunt, 2017), but higher predictivity is achieved with the use of multiple species (Krewski et al., 2010). *In vitro* assays using immortal cell lines can provide a model that can more accurately predict human-specific metabolism and mechanism of toxicity than mammalian studies. However, this data does not reflect a response at the organismal level and is associated with high rates of false-positives and false-negatives, depending on the assay (Pamies and Hartung, 2017; Hunt, 2017). A solution to the problems of cost, time, accuracy, and predictability may be to find an alternative model that is maintained using *in vitro* methods while providing researchers with organismal level data, such as an invertebrate model (Nass and Hamsa, 2007; Tralau et al., 2012).

The use of less expensive and time-consuming models can provide the opportunity to create a pipeline of toxicity testing to identify high-risk compounds faster (Xiong et al., 2017; Rovida et al., 2015). Invertebrate models, such as the free-living nematode *C. elegans*, found in soil and freshwater environments, may offer excellent alternatives that provide data from molecular, organismal, and populations levels (Hoss and Williams, 2009; Hagerbaumer et al., 2015). Toxicity screens with invertebrate models, such as *C. elegans*, can provide a bridge between in vitro and mammalian *in vivo* testing by flagging chemicals with the most potential harm and require further study (Hunt, 2017).

**History of *C. elegans* in Research**

Nematodes, such as *Caenorhabditis elegans* (*C. elegans*), are free-living organisms living in soil, aquatic environments, that play an important role in nutrient cycling and in maintaining environmental quality (Leung et al., 2008). These features make nematodes a good model organism for ecotoxicology studies (Hoss and Weltie, 2007). The first toxicological study using the nematode *Rhabditis elegans* as the test organism was published in 1924 (Honda et al., 1924). Since 1924, a variety of nematode species have been used for studying toxicants and environmental issues, such as *C. elegans*, *Monhystera disjuncta, Plectus acuminatus*, *Panagrellus redivivus*. Of these species, *C. elegans* are the most commonly used nematode for *in vivo* studies (Hägerbäumer et al., 2015).

*C. elegans* emerged as the nematode species of choice following the publication of the seminal genetics paper by Sydney Brenner in 1974. Since 1974, hundreds of studies have been conducted on *C. elegans*, generating extensive knowledge on the anatomy, life cycle, behavior, and genetics of these worms (Hägerbäumer et al., 2015). This knowledge provides a variety of genetic tools (e.g., gene expression, highly conserved signaling pathways, and mutant strains) and endpoints (e.g., development, reproduction, lifespan, locomotion, and behavior) to leverage for *in vivo* toxicity testing (Leung et al., 2008; Corsi, 2006).

In 1988, Williams and Dusenbery published a paper using the nematode *C. elegans* as a predictive animal model to study the toxicity of eight different heavy metal salts. The study reported similar acute lethality, measured as lethal concentration ($LC_{50}$), of the metal salts in *C. elegans* compared to those observed in rodents (Williams and Dusenbery, 1988). Another study in 2004 examining the acute lethality for 15 organophosphates in *C. elegans*, mice, and rats reported comparable results between the organisms (Cole et al., 2004). Furthermore, a recent study used the model organism *C. elegans* to screen over 900 chemicals in the U.S. Environmental Protection Agency's (EPA's) ToxCast™ Phase I and Phase II libraries (Boyd et al., 2016). The study reported similar results in chemical activity in developmental toxicity assays in *C. elegans* and zebrafish, as well as the ability of this data to predict developmental toxicity in rats and rabbits with accuracy (Boyd et al., 2016).

Researchers and regulatory agencies are beginning to shift from observational studies to mechanistic studies that utilize alternative, non-mammalian species with the ability to accurately predict outcomes in humans (Krewski et al., 2010). Currently, the development and validation for alternative predictive toxicology tools is a priority for the Center for Food Safety and Applied Nutrition (CFSAN, 2015; Fitzpatrick and Sprando, 2019). To date, toxicological studies using nematodes have been conducted to examine chemical stressors, such as endocrine disruptors, pharmaceuticals and personal care products, humic substances, polychlorinated biphenyls, phthalates, metal ions, nanoparticles, pesticides, algal toxins, and complex chemical mixtures (Hägerbäumer et al., 2015; Lenz et al., 2017; Lenz et al., 2018). Research in *C. elegans* continues to be conducted because of the ability to collect data at several organizational levels (e.g., molecular, organs, whole organism, and population) in a short period of time (Silverman et al., 2010).

## Toxicity screening with *C. elegans*

In biological research, *C. elegans* have been recognized as an excellent model because of the availability of genetic tools, well-understood biology, transparent bodies, conserved molecular and cellular pathways and ease of culture maintenance or breeding (Hoss and Weltie, 2007; Leung et al. 2008; Haegerbaeumer et al., 2018). *C. elegans* have many genes and signaling pathways that are homologous to mammals - approximately 80% of the identified genes in *C. elegans* have homologues to genes in humans (Leung et al., 2008). These features make this worm a powerful model organism for modelling, drug discovery, and toxicity assessments (Xiong et al., 2017).

### *Molecular Studies in C. elegans*

The 1990s and early 2000s was a time where large scale efforts were aimed at understanding the function of the genome in several vertebrate and invertebrate species (Burdett and van den Heuvel, 2004; Hillier et al., 2005). During this time, the *C. elegans* community collected data on the function, regulation, interaction, and expression of the entire genome of this nematode, identifying highly conserved biological processes found in nematodes and humans (Harris et al., 2004; Corsi et al., 2015; Grove et al., 2018). This fundamental research increased the likelihood that *C. elegans* would be utilized to investigate a never-ending list of biological problems (Corsi, 2006; Corsi et al., 2015). Through the years, many researchers have successfully crossed over to using *C. elegans* as their model organism of choice making this model organism an important tool in biological and toxicological research (Boulin and Hobert, 2013; Tejeda-Benitez and Olivero-Verbel, 2016).

#### Gene expression

A variety of molecular methods have been leveraged to study the molecular response of *C. elegans* to environmental toxicants (Hagerbaumer et al., 2015). There are several examples of *C. elegans* as the model organism for the investigation of the effects of chemicals on the genome using transcriptomic, metabolomic, and proteomic techniques (Sturzenbaum et al., 2012; Kim et al., 2015). These molecular techniques can be applied for the identification of novel genes involved in homeostatsis, detoxification, and the unraveling of the mechanism(s) of toxicity via the utilization of molecular techniques and phenotypic responses (Dexter et al., 2012; Leung et al., 2008). In toxicology, several studies have utilized molecular techniques (e.g., microarrays and qPCR) to examine the effects the effects of toxicants on gene expression and their interactions with other genes (Hagerbaumer et al., 2015; Alexander-Dann et al., 2018).

Researchers have used established molecular tools, such as microarray and qPCR analysis, to examine the expression and mechanism(s) of toxicity-specific stress responsive genes (Alexander-Dann et al., 2018). These studies are used to identify the involvement of defense pathways (e.g., protection against oxidative stress), ion homeostasis, xenobiotic metabolism, hormone regulation, development, and aging (Corsi, 2006). Studies have also been conducted to profile the gene expression of *C. elegans* exposed to ethanol, atrazine, polychlorinated biphenyls, endocrine disrupting chemicals, and polycyclic aromatic hydrocarbons that have been profiled (Custodia *et al.*, 2001; Kwon *et al.*, 2004; Menzel *et al.*, 2007; Reichert and Menzel, 2005).

Green fluorescent protein (GFP)

In the 1960s, the green fluorescence of Aequorea light organs was initially described and led to the isolation and characterization of the two jellyfish proteins responsible for the bioluminescence, a calcium binding protein (aequorin) and a green fluorescent protein (GFP) by Osamu Shimomura (Shimomura, 2008). Following Shimomura's work, Doug Prasher cloned *Aequorea* aequorin in the 1980s and later led collaborations with Martin Chalfie and Roger Tsien (Zimmer, 2009; Chalfie, 2010; Tsien, 2010). Chalfie expressed GFP in *E. coli* and *C. elegans*, while Tsien developed several fluorescent proteins for use in scientific research (Zimmer, 2009). In 2008, Chalfie, Tsien, and Shimomura received the Nobel

Prize in Chemistry for their decades of work discovering and developing GFP, one of the most useful research tools in modern science and medicine (Shimomura, 2008; Chalfie, 2010; Tsien, 2010).

Nowadays, GFP is employed in scientific research to examine different biological functions, including tagging genes for elucidating their expression and localization profiles, monitoring protein trafficking, cell compartmentalization, transformation, virus infection and virus-induced gene silencing, and acting as a biosensor (Rakosy-Tican et al., 2007). Furthermore, fluorescent proteins can also be used to study developmental processes, screen for mutants affecting cell development and function, isolate cells, and characterize protein interactions in vivo (Chalfie et al. 1994; Boulin et al. 2006; Feinberg et al. 2008). The fusing of GFP with specific genes permits the visualization of gene expression *in vivo* via transgenic strains and genome-wide screens (*Conte et al., 2015;* Leung et al., 2008). Determining when and where genes are expressed is often key to determining their function (Hunt-Newbury et al., 2007).

RNAi screens

The discovery of RNA interference (RNAi) mechanisms in *C. elegans* by Fire and colleagues (1998) allowed for the rapid assessment of gene function via genome-wide screens (Agrawal et al., 2003; Kim and Sun, 2007). Fire's work sequencing the nematode genome led to the creation of a publicly available RNAi library that covers approximately 90% of the genes of *C. elegans* (Leung et al., 2008; *Conte et al., 2015*). The RNAi exposure and genome-wide screens contributed to the discoveries of mechanisms of mitochondrial involvement in oxidative stress, programmed cell death, aging, and axon guidance (Boulin et al., 2012; Hagerbaumer et al., 2015; Corsi et al., 2015).

Genome-wide RNAi screen, occurring in both wildtype and knock out mutants, typically assess a variety of parameters at the same time, such as viability, feeding, development, and locomotion, to facilitate the interpretation of the screening results (Koopman et al., 2016). The combining of gene silencing (RNAi) or gene knockout (mutant strains) methods with phenotypic effect screening allows for the verification of the functions of genes (Aagaard and Rossi, 2007; Maine, 2008; Perrimon et al., 2010). Specifically, the use of transgenic strains, the fusing of GFP with genes, permits the microscopic monitoring of gene expression *in vivo* providing a view of the molecular response in specific tissues (*Conte et al., 2015;* Leung et al., 2008). This provides valuable data on the function of genomics that can be leveraged for a variety of scientific research, such as toxicology and pharmaceutical development (Dolphin and Hope, 2006).

Interactome mapping

The mapping of interactions between biomolecules can be useful in understanding their roles and functions, and can be applied to toxicology research (Figeys, 2008). The interest in mapping protein-protein interactions has been reported in several organisms, including *C. elegans* (Mahdavi and Lin, 2007; Eisenhaber et al., 2013). Investigation in *C. elegans* has mapped interactions at the individual scale of biological processes (e.g., vulval development, proteasome, germline, DNA damage response, and dauer formation) and at higher levels of interconnectivity between signaling pathways and the whole proteome (Boy et al., 2012). A study of proteome-scale interaction maps of yeast and *C. elegans* found that 20% of the *C. elegans* genome has detectable orthologs in yeast (Figeys, 2008). Interactome mapping methods are applicable not only to the species of interest, but also for other multicellular species (Rao et al., 2014). Attempts have been made to model the function and dynamics of interactome networks via the integration of various functional genomic approaches, such as expression profiling and genome-wide phenotypic profiling generated through the use of gene knock-outs or RNA interference experiments (Przytycka et al., 2010; Vidal et al., 2011). Information from these studies may be used for applications such as toxicity studies and design of therapeutic drugs (Sun et al., 2012; Boyd et al., 2015).

*Studying the Individual Organism*

*C. elegans* have a number of distinct features that make them an advantageous alternative animal model (Corsi, 2006; Leung et al., 2008). *C. elegans* are simple multicellular organisms that contain approximately 1,000 somatic cells and 302 neurons as adults (Corsi, 2006; Corsi et al., 2015). These worms have short lifespans that allow for rapid experimentation of individual generations or multiple generations (Hoss and Williams, 2009). Additionally, the easy culturing, transparent body, and short generation cycle of *C. elegans* offer advantages for toxicity screening (Leung et al., 2008; Hunt, 2017). These advantages and the previous wealth of knowledge provide a variety of endpoints (e.g., mortality, growth, reproduction, and behavior) that can be utilized for *in vivo* testing (Krewski et al., 2010).

Development

The life cycle of *C. elegans* has several stages (i.e., in utero, ex utero, larval, and adult) (Corsi, 2006). In uterine development is initiated by a sperm fertilizing an oocyte to form an embryo. Embryogenesis of the worm is initiated in utero and completed ex utero, signaled by the hatching of an L1-staged worm consisting of 558 cells (McGovern et al., 2007; Pickett and Kornfield, 2013). In the presence of food, L1-staged larval worms proceed through four stages of development (L1-L4), punctuated by molts, the shedding of the worm's cuticle to allow for increasing body growth (Lazetic and Fay, 2017). During the larval period, a number of blast cells divide, some of which allow for the germline and sexually dimorphic features (e.g., hermaphrodite vulva and male tail) to develop so the animal is able to reproduce when it reaches young adulthood (three days of age). (Corsi et al., 2015; Kipreos and van den Heuvel, 2019).

Reproduction

Once *C. elegans* reach young adulthood (aged 3 days) the hermaphroditic worm has the ability to reproduce (Chasnov, 2013). Hermaphroditic adults have the ability to produce between 200 and 300 progeny and store 10-15 eggs in the uterus (Arur, 2017). The eggs go through 2-3 hours of in utero development prior to the expulsion of one to two eggs through the opening of the vulva (Hart, 2006). The egg-laying behaviors, anatomy, and circuitry components of *C. elegans* are well understood (Schafer, 2005; Collins et al. 2016). Egg-laying events occur approximately every 20 minutes, last for 1-2 minutes, and require proper function of egg-laying muscles (8 uterine muscles and 8 vulval muscles) and neurons (e.g., hermaphrodite-specific motorneurons and ventral nerve cord) (Hart, 2006; Collins et al. 2016). This knowledge of the reproduction of worms can be used to make observations regarding a chemical's potential reproductive toxicity via the use of a variety of endpoints at the molecular and organismal levels, such as transgenic and mutant strains, gene expression, total progeny number, and vulval integrity.

Endocrine system

In toxicology, researchers examine the effects of compounds on the endocrine system. In *C. elegans*, the effects of chemicals on nematodes can only be unequivocally explained as endocrine disruption if the underlying mechanism is identified (Hoss and Weltie, 2007). To achieve this, researchers use our extensive knowledge of hormonal regulation (e.g., hormone metabolism and synthesis, hormone receptors, and signaling pathways) that controls processes, such as molting, reproduction, and cellular metabolism and homeostasis in *C. elegans* (Antebi et al., 2006; Hashmi et al., 2013). Nematodes possess a high number of nuclear receptor genes, 270 genes in *C. elegans* compared to 50 genes in humans, that play important roles regulating sterol metabolism, in utero and ex utero development, molting, reproduction, sex determination, and aging (Brozova et al., 2006; Antebi et al., 2006; Manger and Antebi, 2008). Gene expression studies can also be used to determine which NR genes exhibit increased or decreased activity following an exposure, which allows for additional studies to be conducted using transgenic strains with a green fluorescent marker to observe cellular events occurring in live animals and mutant strains to determine the mode-of-action (Leung et al., 2008).

Aging and Lifespan

*C. elegans* are self-fertilizing hermaphrodites that have a temperature sensitive lifespan, or aging process (Pickett et al., 2013; Xiao et al., 2013). The aging process was not originally considered an actively regulated process; however a major breakthrough in *C. elegans* changed our understanding of the aging process (Tissenbaum, 2015; Uno and Nishida, 2016). Researchers discovered the novel lifespan-regulating genetic pathway, the insulin/insulin-like growth factor-1 (IGF) signaling pathway (Cheng et al., 2005; Kenyon et al., 2011; Altintas et al., 2016). From this research, we know that the IGF signaling pathway is highly conserved in many species, being found in organisms from yeast to humans (van Heemst, 2010). The molecular mechanisms regulating the aging process have garnered a lot of attention in recent decades because of the association of aging and many chronic diseases, such as type 2 diabetes, heart disease, and cancer (Kennedy et al., 2014; Altintas et al., 2016; Tidwell et al., 2017). We know that the aging processes is influenced by genetic and environmental factors, including chemical exposure, xenobiotic metabolism, gene expression, protein translation, and stress response (Uno and Nishida, 2016).

This knowledge of factors influencing aging and the genes involved in the signaling pathway allow researchers to exploit the *C. elegans* model to preform toxicity assays (Hunt, 2017). The utilization of a lifespan assay allows for a large number of worms to be screened and provide researchers with the ability to make observations regarding accumulation of adipose tissue, vulval integrity, and behavior (e.g., locomotion and feeding) (Leiser et al., 2016; Park et al., 2017). Researchers also have the ability to exploit known genetic tools to elucidate that mechanism of toxicity using mutants and transgenic worms along with methods to elucidate gene expression (Bitto et al., 2015; Leung et al., 2015).

*Studying the Impact on a Population*

Researchers can measure population effects for a single generation as well as across generations, due to a large number of progeny and short lifespans of *C. elegans* (Muschiol et al., 2009). The well-understood biology and genetics of the worm can be exploited to observe the effects of chemicals on the life course, from egg to death (Corsi, 2006; Gray and Cutter, 2014). This data can then be used for toxicokinetic-toxicodynamic (TKTD) modeling and dynamic energy budgets (DEB modeling) approaches to gain knowledge on the effects of chemical exposures on population dynamics (Ashauer, 2010; Groh et al., 2015; Gergs et al., 2016). Dynamic models are increasingly used because of their ability to provide information for toxicity predictions of single-chemical and chemical mixtures, such as threshold influx rate for toxicity, maximum duration without toxicity, detoxification rate, and no-effect concentration (Sanchez-Bayo, 2009; Tan and Wang, 2012; Dawson et al., 2014). To date, TKTD and DEB models have been created for nanomaterials, metals, fluoranthene, and uranium using data from studies using *C. elegans* as the model organism (Jager et al., 2014; Margerit et al., 2016; Yang et al., 2017). Moreover, the TBTK/TD approach has been applied to metal toxicity studies using *Daphnia magna* (Tan and Wang, 2012). Although further experimental efforts are needed to confirm these models, these models allow for a more ecologically relevant interpretation of toxicity data for individual chemical exposures, as well as mixtures (Hagerbaumer et al., 2015; Zhang et al., 2018).

**_C. elegans_ - toxicology research and public health policy**

Nematode research has been used for decades and has been increasingly used in recent years to find alternative model organisms to reduce the use of mammals in chemical safety testing, risk assessment, and regulatory decision-making (Norberg-King et al., 2018). The National Institute of Health has requested proposals for studies examining the aging processes and age-related diseases using the model organism model *C. elegans* (NIH, 2016). Furthermore, the National Toxicology Program

Interagency Center for the Evaluation of Alternative Toxicological Methods Interagency Coordinating Committee is developing a list of approved alternative toxicological testing methods and creating a framework for the scientific validation of these alternative methods (Schechtman, 2002; NTP, 2019a). In addition, the U.S. Environmental Protection Agency is exploring invertebrate models for toxicity testing as part of the 21st Century Act (Krewski et al., 2015; EPA, 2018). These changes have resulted in recent local, national, and international meetings with dedicated sessions on alternative animal models, such as *C. elegans*, for public health research and initiatives (Scanlan et al., 2018).

The Food and Drug Administration (FDA) created a document and program that supports the integration of alternative toxicology methods for use in regulatory safety and risk assessments, called the Predictive Toxicology Roadmap (FDA, 2019). Recently, a large-scale assessment of the developmental effects of Phase I and Phase II chemicals in the ToxCast library were shown to cause developmental effects in *C. elegans* that accurately predicts, on the basis of average value of sensitivity and specificity of the assay, known developmental toxicity in rats and rabbits (Boyd et al., 2016). Moreover, last year, the European Food Safety Administration and the European Chemicals Agency (ECHA) published their new guidance on how to identify substances for endocrine disrupting properties, using 5 level systems that include the use of invertebrate models (Andersson et al., 2018). The new guidance uses existing data and epidemiological studies (level 1) and *in vitro* assays (level 2) to aid in informed decision-making for studies falling into levels 3-5, *in vivo* studies. Level 3 studies are used to flag chemicals that pose an adverse risk to environmental and public health using invertebrate and vertebrate models. Chemicals flagged in level 3 are further investigated in levels 4 and 5 *in vivo* studies, providing data for use in creating public health policies (Andersson et al., 2018).

Regulatory agencies in the US, Canada, and Europe are using invertebrate models, such as *C. elegans*, to aid in the investigations of chemicals found in our environmental and consumer products (Krewski et al., 2010). Small non-mammalian animals provide information on the toxicological effects of chemicals on development, the nervous and digestive systems, metabolics, tissue-to-tissue signaling, reproduction, and aging (Planchart et al., 2016; NTP, 2019a; NTP, 2019b). Many pathways involved in organismal, endocrine function, and neural development are highly conserved in invertebrates and mammals (Leung et al., 2008). Once validated, *in vivo* assays using such phylogenetically lower species can support new methods for risk assessment that can be used by regulatory agencies (Krewski et al., 2010; FDA, 2019; NTP, 2019a).

**Promising avenues for toxicity testing in *C. elegans***

*C. elegans* were first used as a predictive toxicology model in 1988 for an acute lethality assay (Williams and Dusenberry, 1988). Since then, numerous studies have used this model organism to examine toxicants for their effects at molecular, organismal, and population levels using a variety of sophisticated techniques (Choi, 2008; Hunt 2017). These techniques have been used to profile potential toxicants, including endocrine disruptors, ethanol, humic substances, polychlorinated biphenyls, phthalate, bacterial toxins, metal ions and nanoparticles, and complex chemical mixtures in contaminated sediments (Hagerbaumer et al., 2015). This model organism is a promising tool for the testing of emerging contaminants, designed therapeutics, and chemicals used in personal care and consumer products (Corsi et al., 2015).

*Cyanopeptides*

Over the past decade, the occurrence of cyanbacterial blooms have increased in frequency and magnitude worldwide and pose a potential threat to environmental and public health, as well as local economies (Pick, 2016; Wilkinson et al., 2018; Kimambo et al., 2019). These blooms can produce highly toxic secondary metabolites (cyanopeptides) known as cyanotoxins that have been in drinking water and food sources at concentrations reaching mg/L, posing a significant public health risk (Gkelis et al., 2015; Kurmayer et al., 2016). In recent years, the detection of cyanotoxins in drinking water and food sources has resulted in the shutting down of drinking water facilities in Oregon and Ohio, as well as fisheries in North and Central America (Michalak, 2016; MDH, 2017). As a result, increased occurrences of cyanobacterial blooms may increase the challenge of maintaining safe drinking water, food, and recreational aquatic environments (Murphy et al., 2012; Miller et al., 2017).

The most commonly known cyanopeptide is microcystin (MC); however there are more than 600 identified cyanopeptides (Gkelis et al., 2015). MC occurs in waterbodies found in temperate climates and is known to pose a risk to environmental and human health (Chorus and Fastner, 2001). For this reason, the World Health Organization set guideline values for detectable MC in drinking and recreational water, as well as proposed implementation measures for monitoring and control of MCs (WHO, 2008). Additionally, other cyanopeptides (e.g., microginins, cyanopeptolins, anabaenopeptins, anabaenopeptilides, microvirdins, and nostophycins) are commonly detected in the environment; however little is known about the potential risk many of these peptides may pose to the public and environment. (Welker and Von Dohren, 2006).

Recent studies have found that cyanopeptolins and anabaenopeptins are often produced with MCs and in some cases are detected at equal or higher concentrations than MCs (Beversdorf et al., 2017, 2018; Janssen, 2019; Larsen et al., 2019). For example, anabaenopetin-B and –F were measured at 6.56 µg/L combined in Lake Koshkonong, Wisconsin, while total microcystin was measured at an average concentration of 0.65 µg/L (Beversdorf et al., 2017). Furthermore, the concentration of anabaenopeptins in blooms have been reported to exceed 1000 µg/L in freshwater bodies in Greece (Gkelis et al., 2015). The toxicological effects of most cyanopeptides are unknown, leaving them unregulated and without published provisional guideline values for drinking water and recreational environments (Larsen et al., 2019). This lack of knowledge requires immediate attention as cyanobacterial blooms and cyanopeptide detection increase in frequency.

*Antimicrobials*

Antimicrobials, such as triclosan (TCS) and triclocarban (TCC), are used in numerous personal care products, consumer products, and medical devices (i.e., cosmetics, food packaging, plastics, children's toys, clothing, building materials, textiles, and medical devices) (Halden, 2014). TCS and TCC enter the environment through wastewater containing personal care products (i.e., soaps, toothpaste, hair care products, and cosmetics) that are washed down our drains every day (Pycke et al., 2014). The frequent use and ubiquitous detection of TCS and TCC have raised concerns regarding the toxicological effects these chemicals have on human health and the environment (Halden, 2016). These growing concerns have led to numerous *in vivo*, *in vitro*, and epidemiological studies on TCS and TCC, such as their degradation products, bioaccumulation in aquatic plants and animals, partitioning into blood and breast milk, contribution to antibiotic resistance, and increased allergen sensitivity (Halden et al., 2017).

In the fall of 2016, the FDA banned 19 chemicals (e.g., TCS, TCC, other halogenated aromatics, and methylbenzethonium chloride) from antibacterial soaps (FDA, 2016). This bold step forward was the result of decades of research and pressure from the scientific community and general public to regulate these chemicals. While these chemicals are no longer used in soaps, there are a wide variety of other products in which these chemicals are commonly used (Halden, 2014). Furthermore, the ban postponed a final ruling on chemicals that there was little toxicology data for at the time, such as benzalkonium chloride, benzethonium chloride, and chloroxylenol (FDA, 2017).

Following this ruling by the FDA, a group of more than 200 scientists and medical professionals released a statement calling for further restrictions on the use of antimicrobials, as well as addressed gaps in our knowledge regarding the adverse effects of exposure to two antimicrobials, triclosan (TCS) and triclocarban (TCC) (Halden et al., 2017). Their statement summarized decades of research and raised concerns regarding the long-term health and ecological impacts of antimicrobials and the need for greater transparency during product development and toxicity testing before a chemical is used in a commercial product (Halden et al., 2017).

A way in which early toxicity testing can be performed early in the production of chemicals and products is through the use of a high-throughput animal model, such as an invertebrate model. The use of *C. elegans*, or another invertebrate model, can provide a quick screen for new chemicals moving forward. Faster screening platforms will allow researchers, regulatory agencies, and companies the ability to flag chemicals that pose a potential risk to environmental and human health earlier in the process. This will allow concerns to be addressed before toxic and environmentally persist enter our environment.

**Conclusion**

The unique features of invertebrates make these organisms excellent animal models in toxicological research. The available methods, well-understood biology, and extensive genetic information makes the nematode *C. elegans* an excellent model to screen potential toxicants and study the role of specific gene(s) in toxicological processes and gene-environment interactions. *C. elegans*-based assays do not have the same costs as *in vivo* vertebrate models and allow for the testing of hypotheses with an intact organism. Thus, *C. elegans*-based assays provide an excellent complement to *in vitro* and *in vivo* models. The use of an invertebrate model will allow for faster screening of chemicals that will allow for appropriate stakeholders to address concerns earlier in the risk assessment process. Ideally, this will eliminate the use of high risk chemicals and limit their introduction into to the environment.

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CHAPTER 2:

# Anabaenopeptins and cyanopeptolins induce systemic toxicity effects in a model organism the nematode *Caenorhabditis elegans*


**Abstract**

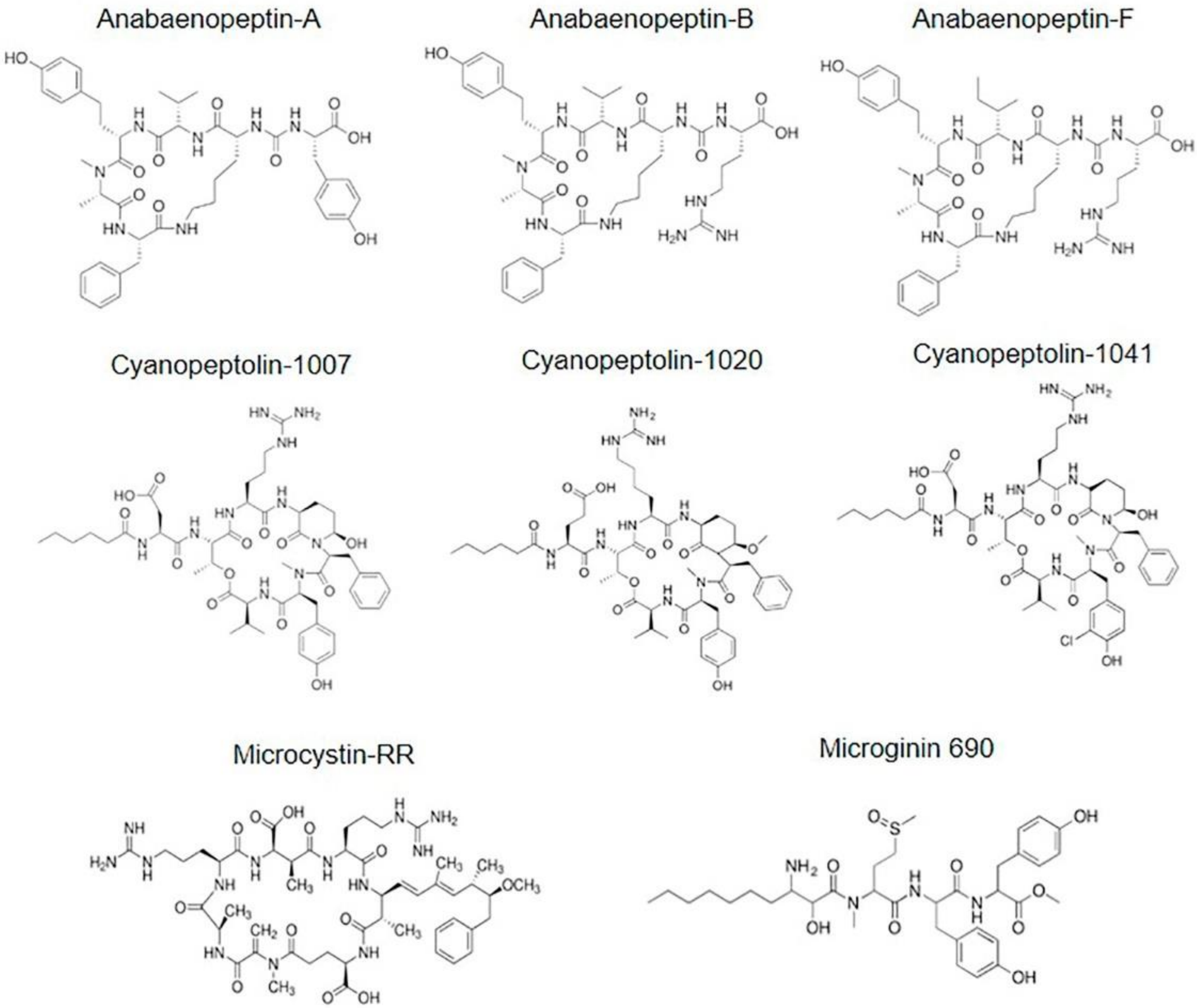
Cyanobacterial blooms represent a significant risk to environmental and human health due to their production of toxic secondary metabolites, cyanopeptides. Anabaenopeptins and cyanopeptolins are cyanopeptides increasingly detected in surface waters at concentrations exceeding regulatory toxicity levels for other cyanotoxins such as microcystins. Yet their toxicity to aquatic organisms are not well understood. Here we assessed the toxicological effects of three anabaenopeptins (AP-A, AP-B, and AP-F) and three cyanopeptolins (CYP-1007, CYP-1020, and CYP-1041) to a model organism the nematode *Caenorhabditis elegans*. Examined toxicity endpoints included reproduction, hatching time, growth rate, lifespan, and age-related vulval integrity. Microcystin RR (MC-RR) and microginin 690 were also included in the study for comparisons. At an identical mass concentration (10 µg/L, corresponding to a molar concentration ranging 0.01–0.014 µM depending on the specific peptide), anabaenopeptins (APs) showed the greatest toxicity among all cyanopeptides tested. APs decreased worm reproduction by 23%–34% and shortened worm lifespan by 5 days (a 30% reduction) compared to the controls. APs also induced a remarkable age-related vulval integrity defect (Avid phenotype) in the worm, where over 95% of exposed worms developed the phenotype, compared to a less than 15% in control worms. CYPs showed similar toxicity as MC-RR, and Microginin 690 was the least toxic. These findings suggest that APs and CYPs may pose significant health risks to aquatic organisms. More toxicological studies of these cyanopeptides using different species across different trophic levels are needed to gain a thorough understanding of their potential impact on ecological systems and human health.

## Introduction

Cyanobacterial blooms have increased in frequency and magnitude across the globe over the past decade (Pick, 2016). These blooms have the potential to wreck havoc on environmental and public health, as well as local economies (Wilkinson et al., 2018) as they can produce highly toxic secondary metabolites (cyanopeptides) known as cyanotoxins. Cyanotoxins have been detected in drinking water and food sources at concentrations up to mg/L (Gkelis et al., 2015, Kurmayer et al., 2016), posing a significant risk to the public's health. In August 2014, an algal bloom contaminated the drinking water of Toledo, Ohio forced nearly half a million people to drink bottled water for three days (MDH, 2017). A bloom of *Pseudo-nitzschia* containing the neurotoxin domoic acid occurred off the west coast, shutting down fisheries from Baja California in Mexico up to Alaska in 2015 (Michalak, 2016). More recently in May 2018, Salem in Oregon shut down drinking water for kids because of algal toxin contamination caused by algae blooms in Detroit Reservoir (Michalak, 2016). As a result, the maintenance of safe drinking water and recreational aquatic environments may become more challenging with the increased occurrences of cyanobacterial blooms (Murphy et al., 2012, Miller et al., 2017).

Cyanotoxins can be classified as peptides or alkaloids based on their chemical structures. They may also be classified as hepatoxins, neurotoxins, or dematotoxins based on their mechanisms of toxic action in vertebrates, especially mammals (Ferrao-Filho and Kozlowsky-Suzuki, 2011). Peptides are the largest group of cyanotoxins and there have been more than 600 cyanobacterial peptides described to date (Gkelis et al., 2015). Microcystins (MCs) are among the first discovered and described cyanopeptides, occurring abundantly in waterbodies in temperate climates, and can pose significant health risks to livestock, wildlife, fishes, and humans (Chorus and Fastner, 2001). The WHO has proposed implementation measures for monitoring and control of MCs and determined guideline values for drinking and recreational waters (WHO, 2008). Other commonly found bioactive peptides include

Figure 2.1. Structures of the cyanopeptides studied.



microginins, cyanopeptolins, anabaenopeptins, anabaenopeptilides, microvirdins, and nostophycins (Welker and Von Dohren, 2006). Anabaenopetins (APs) and cyanopeptolins (CYPs) are cyclic nonribosomal oligopeptides produced by a broad range of cyanobacterial species. APs are characterized by a ring of five amino acid residues including a conserved lysine. CYPs have a six amino acid residue ring structure, a conserved 3-amino-6-hydroxy-2-piperidone (AHP) residue and a side chain with variable length (Fig. 2.1). More than 100 different APs and CYPs have been reported (Chilipala et al., 2012, Cerasino et al., 2017), and they are commonly detected along with MCs. A recent study has found that AP concentrations in blooms can exceed 1000 μg/L in freshwater bodies in Greece (Gkelis et al., 2015).

Cyanopeptides induce toxicity to animals and humans through diverse mechanisms, ranging from hepatotoxic and cytotoxic effects to the inhibition of protein synthesis. MCs are the most commonly observed cyanobacterial liver toxins globally (Miller et al., 2017). They covalently bind to and inhibit protein phosphatase type 1 or 2A in liver cells and may also inhibit other proteins and enzymes. MCs are among the most extensively studied algal toxins due to their high toxicity. They have been suspected as the culprit of numerous animal and human poisonings across the globe (Backer et al., 2013, Weirich and Miller, 2014, Trevino-Garrison et al., 2015). Adverse effects from ingestions of MCs have been observed in various aquatic organisms. Ingestion of MC-producing cyanobacteria resulted in lethal poisoning in *Daphnia galeata*, at an intake of 10.2 ng of MC per 1 mg body weight (Rohrlack et al., 2005). Long-term low dose exposure to MCs in *Daphnia magna* resulted in accumulation of the cyanotoxin in the organism and the phosphatase enzyme activity was also inhibited (Chen et al., 2005). Accumulation of MC-LR in a gastropod pulmonate *Lymnaea stagnalis* following aqueous exposure, accompanied by a strong decrease in egg production in adult organisms was also reported (Gérard et al., 2005). Toxicity of MCs to several fish species have also been reported, and observed effects ranged from mortality to developmental abnormalities, depending on the cyanotoxin concentrations (Zanchett and Oliveira-Filho, 2013).

In contrast, studies on ecological toxicity of APs and CYPs are scarce. Both APs and CYPs are known inhibitors of serine proteases and protein phosphatases (Spoof et al., 2016, Gademann and Portmann, 2008, Gademann et al., 2010), which are enzymes responsible for the regulation of several vital physiological and metabolic processes. Most of these bioactive peptides have been labelled as non-toxic, although the full effect of these peptides individually or in mixtures to the environmental biota is largely unknown (Miller et al., 2017). A recent study discovered that CYP-1020 induced neurotoxicity in a zebrafish model and inhibited human kallikrein and trypsin in the low pico- to nano-molar range (Gademann et al., 2010, Faltermann et al., 2014). CYP-1020 also showed a similar LC50 (8.8 µM) against fairy shrimp (*Thamnocephalus platyurus*) with MC-LR (10.8 µM) (Gademann et al., 2010). Thus, these previously considered "non-toxic" cyanopeptides may represent emerging cyanotoxins, whose toxicity effects to aquatic organisms warrant immediate attention.

In this study, we investigated the toxicity effects of three APs (AP-A, AP-B, and AP-F) and three CYPs (CYP-1007, CYP-1020, and CYP-1041) to a model organism the nematode *C. elegans.* MC-RR and microginin-690 were also included in the study for comparisons. The former belongs to the group of MCs with known high toxicity potency and the later is much less toxic based on existing literature (Zanchett and Oliveira-Filho, 2013). *C. elegans* is a free-living nematode found in soil and freshwater environments (Hoss and Williams, 2009). It is an excellent model organism in toxicology due to the availability of genetic tools, well-understood biology, transparent body, conserved molecular and cellular pathways with humans, and ease of culture in the laboratory (Leung et al., 2008, Haegerbaeumer et al., 2018). It has been used for toxicity assessment for a broad range of chemical contaminants at environmentally relevant concentrations including metals (Wu et al., 2012), engineered nanoparticles (Zhang et al., 2011), and organic pollutants (Zhou et al., 2016). Here we assessed the toxicity effects of these cyanotoxins to the worm using endpoints of hatching rate, growth rate, reproduction, lifespan, and vulval integrity. We selected a concentration of 10 µg/L, which is a WHO recommended guideline for microcystins for recreational water (WHO, 2003), for our study. Our goal is to illustrate the toxicity effects of these understudied cyanopeptides at a regulatory level that has been established for well-studied cyanotoxins. We expect findings from this study will help reveal the baseline toxicity of these APs and CYPs and inform future studies for further understanding of their environmental and human health impact and development of necessary guidelines to protect human and ecosystem health.

## Materials and methods

### Chemicals

The cyanotoxins, anabaenopeptin A (AP-A) (>95% purity), anabaenopeptin B (AP-B) (>95% purity), anabaenopeptin F (AP-F) (>95% purity), cyanopeptolin 1007 (CYP-1007) (>95% purity), cyanopeptolin 1020 (CYP-1020) (>95% purity), cyanopeptolin 1041 (CYP-1041) (>95% purity), and microginin 690 (>95% purity) were purchased from MARBIONC (Wilmington, NC, USA). Microcystin RR (MC-RR) (>90% purity) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Stock solutions (10,000 μg/L) were prepared by suspending the cyanotoxins in methanol (MeOH). K-medium (51 mM NaCl, 32 mM KCl, pH 6.8) was used as the diluent to create the exposure concentration (10 μg/L) used for all sub-lethal exposure assays. The final MeOH concentration in all working solutions was 0.1%.

### *C. elegans* strain

The *C. elegans* strain N2 Bristol (wildtype) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). Wildtype nematodes were maintained following a standard protocol: maintained on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 and stored at 20 °C (Stiernagle, 2006). Age-synchronized worm populations were used for all sub-lethal assays. The generation of an age-synchronized population was achieved by collecting eggs from gravid adult *C. elegans* and bleaching with 1% NaClO and 0.013M NaOH solution (Donkin and Williams, 1995).

**Reproduction**

Reproduction tests were performed using age-synchronized L4-stage nematodes. L4 worms were placed on OP50 seeded NGM plates which have been amended with 150 µL of K-medium (negative control), MeOH (vehicle control), or 10 µg/L of cyanotoxins (APs, CYPs, MC-RR, or microginin 690). NGM plate preparation for exposures was previously described by Lenz et al. (2017). One L4-stage worm was added to each plate and kept at 20 °C for the duration of the exposure (approximately six days in total). Six worms (6 plates) were used for each test concentration. For each plate, the parent worm was transferred to a fresh plate every other day (2–3 total transfers over approximately six days), and the number of progeny (eggs and larval worms) was recorded. Following the reproductive cycle, the average number of progeny per worm was calculated by adding the number of progeny from all plates of an exposure concentration.

**Hatching time and growth**

The experimental method for hatching time, the time from egg disposition to hatching (Muschiol et al., 2009), has been previously described by Lenz et al. (2017). Eggs from age-synchronized 3-day old worms were collected and prepared using a standard protocol (Donkin and Williams, 1995). Approximately 300 eggs were placed on an OP50 plate amended with 300 µL of K-medium (negative control), MeOH (vehicle control), or 10 µg/L of cyanotoxin (APs, CYPs, MC-RR, or microginin 690). The number of eggs hatched was recorded every 3 h until all eggs hatched. The hatched larval worms were then examined for growth.

To assess growth, the size (body length) of worms were measured at 18, 24, 48, and 72 h of upon egg collection during the developmental process (L1-L4). The body length of the worm was defined as the length from the opening of the mouth (anterior end) to the tip of the tail (posterior end) (Morck and Pilon, 2006). Growth of the wildtype worm has been extensively described in the literature (Byerly et al., 1976). Worm images were taken for each exposure condition in the above described time points using a Nikon AZ100 microscope and NIS-Elements BR 3.2 software. Images were analyzed using Zen 2011 software (Carl Zeiss Microscopy) to obtain length measurements of all worms. Approximately 100 worms were measured at each time point for each exposure condition. The average body length was then calculated.

**Lifespan and vulval integrity**

Lifespan experiments followed a standard protocol, using NGM plates seeded with OP50 and 0.2 mM 5-fluoro 2-deoxyuridine (FUdR) to inhibit progeny production (Pluskota et al., 2009). Plates were amended with 150 µL of K-medium (negative control), MeOH (vehicle control), or 10 µg/L of cyanotoxins. Fifty worms were placed on each plate, and two plates were used for each exposure (totaling 100 worms per exposure per replicate). Every other day, the worms were examined, and the dead ones were removed from the plate and the number of death was recorded. Death was defined as failure to respond to an external stimulus, a gentle tapping of the anterior and posterior ends with a platinum picker. Worms that burrowed in the agar or crawled off the plates were excluded from the data recording. This process continued till all the worms died, which is usually around 2.5–3.5 weeks from the beginning of exposure, depending on the exposure conditions.

The worms were examined for age-related vulval integrity defects (Avid) during lifespan experiments, according to the criteria described by Leiser et al. (2016). Avid is characterized by a loss of integrity of the vulva occurring in post-mitotic hermaphrodites and is classified into three types based on the severity of the defects. Avid I is defined as the presence of a herniated vulva. Avid II is defined as the presence of the tearing or rupturing of the herniated vulva with visible excretion of material. Avid III is defined as the exploded vulva, indicated by the presence of muscle and intestines outside the body. The phenotype typically starts to appear when the worms are 8–9 days old. Worm images were taken every other day during the lifespan experiments using a Nikon AZ100 microscope and NIS-Elements BR 3.2 software. Upon the death of the worms, they were scored for the above-described Avid phenotypes and the overall frequency of the phenotypes (Avid I, II, and III combined) was calculated.

**Data analysis**

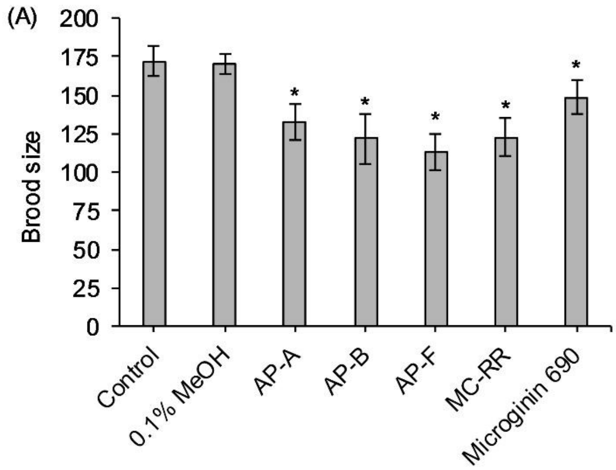
All sub-lethal assays were repeated two times. One-way analysis of variance (ANOVA) followed by pairwise multiple comparisons (Holm-Sidak method) were performed for comparisons between individual cyanotoxins in terms of impact on reproduction, growth, and vulval integrity. Lifespan data were analyzed using Kaplan-Meier survival analysis and a log-rank test was used for the estimation of mean lifespan (IBM SPSS Statistics, Armonk, NY). The average hatching time (with 95% confidence intervals) under different exposure conditions was calculated and compared using ANOVA analysis (IBM SPSS Statistics, Armonk, NY).

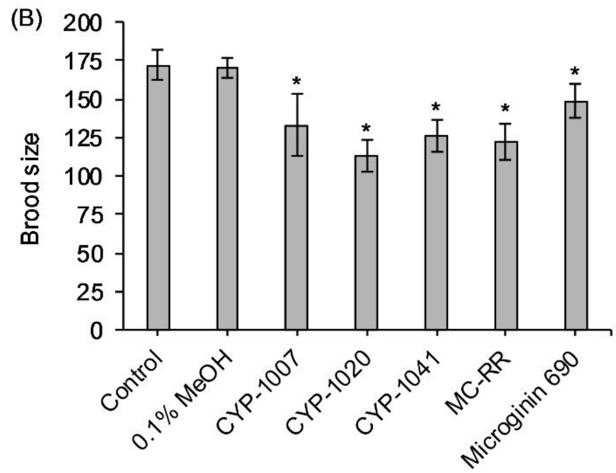
**Results**

**Impact on reproduction**

APs, CYPs, and MC-RR all showed significant adverse impact on worm reproduction (Fig. 2). Worms exposed to the three APs at 10 μg/L (0.012 μM) had an average brood size ranging from 133 ± 11 to 114 ± 11, significantly lower than the brood size of 172.1 ± 10 in controls (Fig. 2.2A, one-way AONVA, $p < 0.001$). This represents a decrease of 23%–34% from the control. Worms exposed to the three CYPs at 10 μg/L (0.01 μM) had an average brood size of 133 ± 20 to 113 ± 10, significantly lower than the

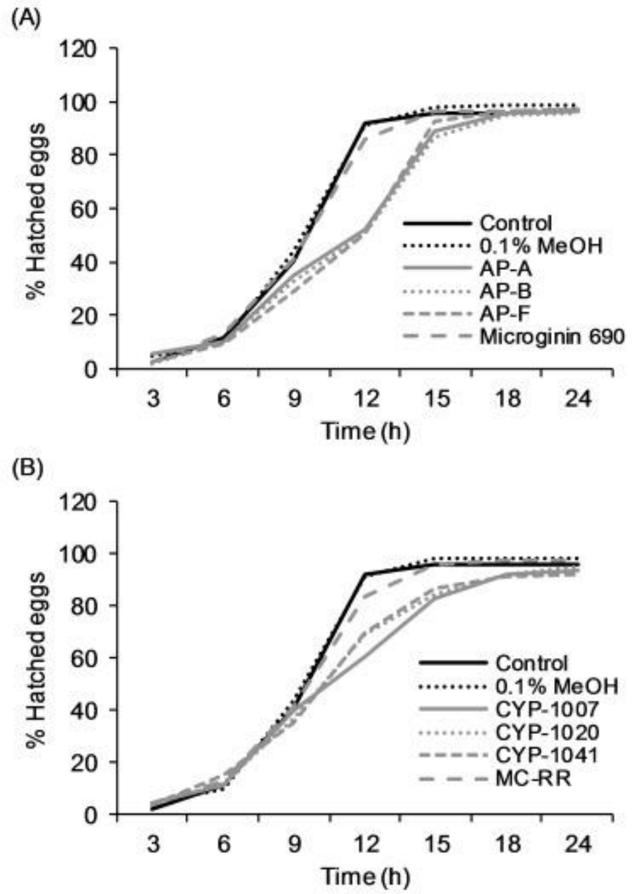
Figure 2.2. Impact of cyanopeptides (10 µg/L) on *C. elegans* reproduction.




(A) Anabaenopeptins (AP-A, AP-B, and AP-F). (B) Cyanopeptolins (CYP-1007, CYP-1020, and CYP-1041). Error bars represent standard deviation (n = 12, two independent experiments). "*" indicates a significant difference from controls (one-way ANOVA, $p < 0.001$).

Figure 2.3. Impact of cyanopeptides (10 μg/L) on *C. elegans* hatching time.




(C)

| Exposure | Average hatch time (h, 95% CIs) | Change from control (h) | % Change |
|---|---|---|---|
| Control | 10.1 (9.9, 10.3) | - | - |
| 0.1% MeOH | 10.2 (10.0, 10.4) | 0.1 | 0.6% |
| AP-A | *11.7 (11.4, 12.1) | 1.6 | 16.1% |
| AP-B | *12.1 (11.8, 12.4) | 2 | 19.9% |
| AP-F | *12.1 (11.8, 12.4) | 2 | 20.0% |
| CYP-1007 | *11.5 (11.1, 11.8) | 1.4 | 13.3% |
| CYP-1020 | *11.4 (11.1, 11.8) | 1.3 | 12.8% |
| CYP-1041 | *10.9 (10.6, 11.3) | 0.8 | 8.3% |
| MC-RR | 10.4 (10.1, 10.6) | 0.3 | 2.6% |
| Microginin 690 | 10.5 (10.2, 10.7) | 0.4 | 3.4% |

(A) Anabaenopeptins (AP-A, AP-B, and AP-F) and microginin 690; (B) cyanopeptolins (CYP-1007, CYP-1020, and CYP-1041) and MC-RR. (C) Average hatching time and percent difference compared to controls. “*” indicates a significant difference from controls (one-way ANOVA, $p < 0.05$).

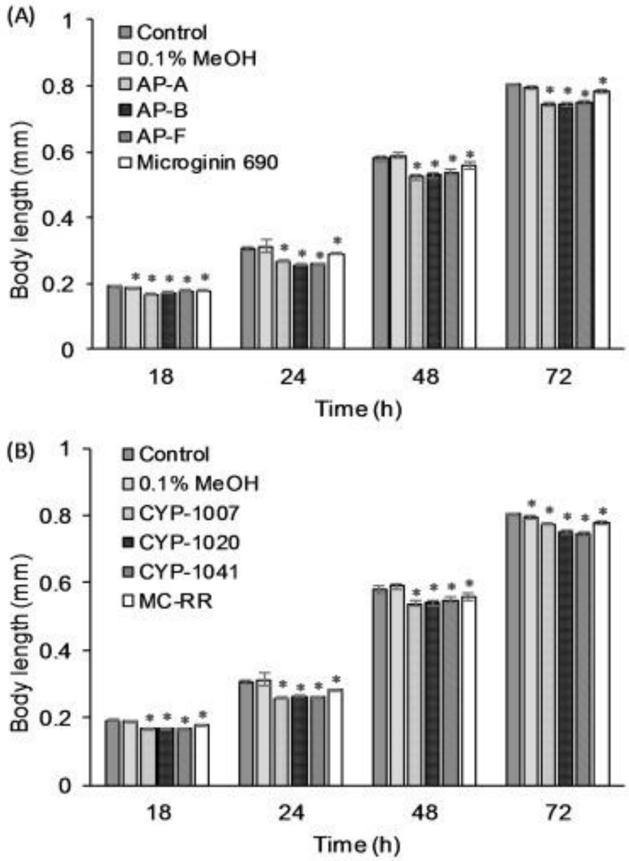
controls (Fig. 2.2B, one-way AONVA, $p < 0.001$). Worms exposed to MC-RR (10 µg/L, 0.01 µM) had a brood size of 123 ± 12 (29% decrease from the control), and worms exposed to microginin 690 (10 µg/L, 0.014 µM) had a brood size of 149 ± 11 (13% decrease from the control). Further multiple comparisons (Holm-Sidak method) indicated that at an identical mass concentration 10 µg/L, MC-RR had similar impact on the worm's brood size as APs or CYPs, whereas microginin 690 had significantly lower impact than APs or CYPs ($p < 0.05$).

**Impact on worm hatching and growth**

We examined the effects of APs and CYPs on embryogenesis, the time from fertilization to egg hatching, using the endpoint hatching time (Lenz et al., 2017). Typically, ex utero development of the worm from egg to hatching takes ∼9–10 h under 20 °C (Altun and Hall, 2018). As can be seen from Fig. 2.3A, by the time of 12 h, over 90% of control eggs or eggs exposed to Microginin 690 (10 µg/L, 0.014 µM) had hatched, whereas less than 50% of eggs exposed to the three APs (10 µg/L, 0.012 µM) had hatched, indicating a substantial delay in hatching. The calculated average hatching time for the control eggs was 10.1 h (95% CI: 9.9, 10.3), and for the APs-exposed worms were 11.7 (95% CI: 11.4, 12.1) to 12.1 (95% CI: 11.8, 12.4) h (Fig. 2.3C), showing an approximate 2 h delay in hatching. Similarly, the three CYPs (10 µg/L, 0.01 µM) delayed the worm's hatching, but to a less extent than APs. Compared to over 90% of hatching at 12 h in control eggs, eggs exposed to CYPs had a 60–70% of hatching rate at 12 h (Fig. 2.3B). The average hatching time of eggs exposed to CYPs were 10.9 (95% CI: 10.6, 11.3) to 11.5 (95% CI: 11.1, 11.8) h. MC-RR or Microginin 690 exposed eggs showed similar hatching time as control worms (Fig. 2.3C).

The hatched larval worms were further examined through the developmental process (L1-L4) to determine if exposure to these cyanotoxins have an impact on the worm's growth during development. As shown in Fig. 2.4A and B, exposure to APs or CYPs caused growth delay of the worms as indicated by

Figure 2.4. Impact of cyanopeptides (10 µg/L) on *C. elegans* growth rate.

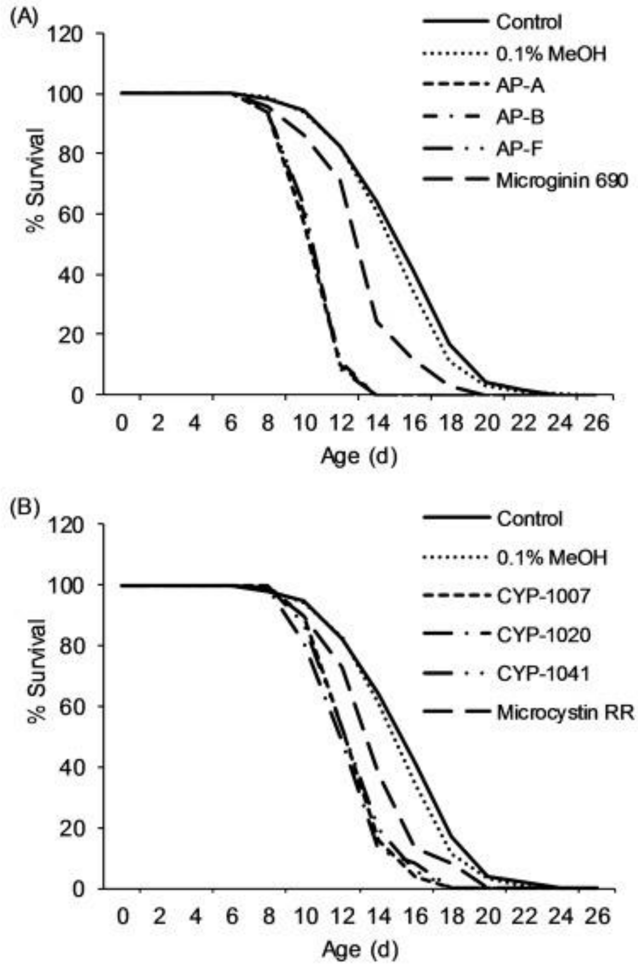



(C)

| Exposure | Time (h) | | | |
|---|---|---|---|---|
| | 18 | 24 | 48 | 72 |
| Control | - | - | - | - |
| 0.1% MeOH | -1.8% | 2.7% | 1.7% | -1.2% |
| AP-A | -13.3% | -12.3% | -9.4% | -7.2% |
| AP-B | -10.0% | -15.7% | -8.5% | -7.5% |
| AP-F | -7.5% | -15.0% | -7.0% | -6.7% |
| CYP-1007 | -13.3% | -15.2% | -7.0% | -3.7% |
| CYP-1020 | -12.1% | -13.3% | -6.8% | -6.9% |
| CYP-1041 | -13.1% | -14.4% | -5.3% | -7.4% |
| MC-RR | -8.4% | -7.8% | -3.5% | -3.1% |
| Microginin 690 | -7.7% | -5.1% | -3.7% | -2.8% |

(A) Anabaenopeptins (AP-A, AP-B, and AP-F) and microginin 690; (B) cyanopeptolins (CYP-1007, CYP-1020, and CYP-1041) and MC-RR. (C) Percent difference compared to controls. “*” indicates a significant difference from controls at each time point (one-way ANOVA, $p < 0.05$).

Figure 2.5. Impact of cyanopeptides (10 µg/L) on *C. elegans* lifespan.




(C)

| Exposure | Average lifespan (95% CIs; d) | Change from control (d) | % Change |
|---|---|---|---|
| Control | 16.1 (15.7, 16.4) | - | - |
| 0.1% MeOH | 15.7 (15.4, 16.0) | -0.4 | -2.5% |
| AP-A | *11.2 (11.0, 11.4) | -4.9 | -30.4% |
| AP-B | *11.3 (11.1, 11.5) | -4.8 | -29.8% |
| AP-F | *11.3 (11.1, 11.5) | -4.8 | -29.8% |
| CYP-1007 | *13.3 (13.0, 13.5) | -2.8 | -17.4% |
| CYP-1020 | *13.0 (12.7, 13.3) | -3.1 | -19.3% |
| CYP-1041 | *13.3 (13.0, 13.6) | -2.8 | -17.4% |
| MC-RR | *14.4 (14.0, 14.8) | -1.7 | -10.6% |
| Microginin 690 | *13.9 (13.5, 14.2) | -2.2 | -13.7% |

(A) Anabaenopeptins (AP-A, AP-B, and AP-F) and microginin 690; (B) cyanopeptolins (CYP-1007, CYP-1020, and CYP-1041) and MC-RR; (C) average lifespan and percent difference compared to controls. "*" indicates a significant difference from controls (log-rank test, $p < 0.05$).

decreased body length in exposed worms than control worms at each time point (one-way ANOVA, $p < 0.05$). For example, at 24 h, the average body length of larval worms exposed to APs was decreased by 12.3%–15% compared to the controls. This decrease in body length seemed to be ameliorated as the development progresses. At 48 h, the difference in body length between AP-exposed and control worms was 7%–9.4%. At 72 h, when the worms reached young adult stage, the AP-exposed worms were 6.7%–7.5% smaller in body size (length) than the controls (Fig. 2.4C). At an identical mass concentration of 10 μg/L, CYPs had a similar impact on the worm growth as APs (Fig. 2.4C). MC-RR and microginin 690 had less effect on the worm growth, indicated by a decrease in body length by 7.8% and 5.1%, respectively, at 24 h. At 72 h, this difference was 3.1% and 2.8%, respectively.

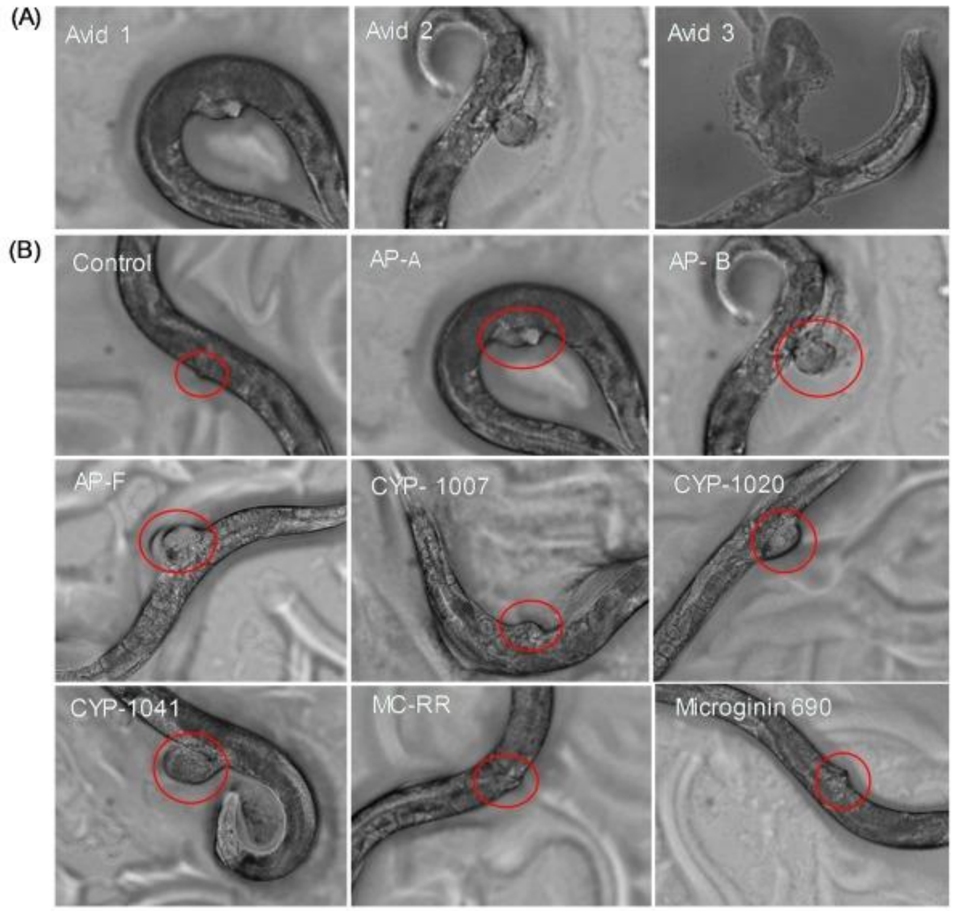
**Impact on lifespan**

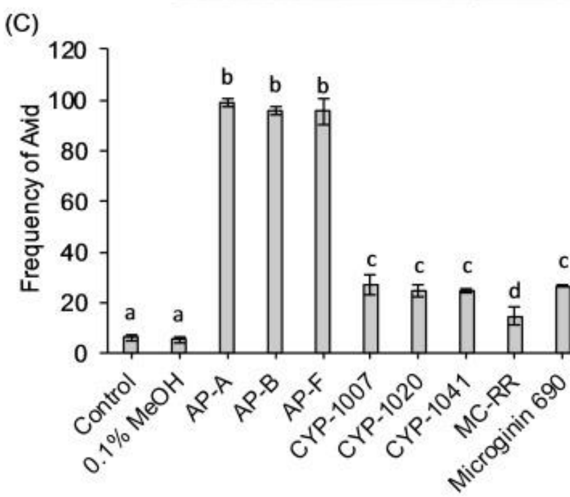
Both APs and CYPs substantially decreased the average lifespan of the worm (Fig. 2.5A and B). Worms exposed to three APs (10 μg/L, 0.012 μM) had a mean lifespan around 11 days (95% CI: 11, 11.5) as compared to 16 days (95% CI: 15.7, 16.4) of control worms, corresponding to a 30% decrease from the control. Worms exposed to three CYPs (10 μg/L, 0.01 μM) had a mean lifespan around 13 days (95% CI: 12.7, 13.6), corresponding to an 18% decrease from the control. MC-RR and microginin 690 decreased the average lifespan by 1.7 days and 2.2 days, corresponding to 11% and 14% decrease from the control, respectively (Fig. 2.5C).

**Impact on vulval integrity**

Following exposure to all cyanotoxins, worms showed an Avid phenotype. Using the classification criteria described by Leiser et al. (2016), we found that all three APs (10 μg/L, 0.012 μM) induced Avid (I, II, and III collectively) in >95% of the exposed worms compared to less than 10% in control worms (Fig. 2.6A–C, one-way ANOVA, $p < 0.001$). Specifically, Avid I was the dominant

Figure 2.6. Impact of cyanopeptides (10 µg/L) on vulval integrity in *C. elegans*.




(D)

| Exposure | Frequency | | | |
|---|---|---|---|---|
| | Avid 1 | Avid 2 | Avid 3 | Total |
| Control | 5% | 1% | 0% | 6% |
| 0.1% MeOH | 5% | 1% | 0% | 5% |
| AP-A | 60% | 38% | 2% | 99% |
| AP-B | 58% | 38% | 1% | 96% |
| AP-F | 50% | 45% | 1% | 96% |
| CYP-1007 | 18% | 8% | 2% | 27% |
| CYP-1020 | 17% | 7% | 1% | 25% |
| CYP-1041 | 17% | 7% | 1% | 25% |
| MC-RR | 14% | 1% | 0% | 15% |
| Microginin 690 | 20% | 7% | 0% | 27% |

(A) Classification of age-related vulval integrity defects (Avid) according to the criteria defined by Leiser et al. (2016); (B) representative images of worms showing Avid phenotype upon exposure to different cyanopeptides; (C) frequency of Avid phenotype in worms exposed to different cyanopeptides; (D) frequency of Avid breakdown by different Avid classification. Error bars represent standard deviation. Different letters indicate significant differences (one-way ANOVA, Holm-Sidak comparison, $p < 0.05$).

phenotype, accounting for 50–59.5% within the >95% of incidence (Fig. 2.6D). Exposure to CYPs (10 μg/L, 0.01 μM) caused an Avid incidence of 25%, significantly higher than controls (one-way ANONA, $p < 0.05$) but much less than the APs. Microginin 690 (10 μg/L, 0.01 μM) induced Avid at a comparable rate (26.5%) to the CYPs, while MC-RR induced Avid to a less extent (14.5%) (Fig. 2.6C). All Avid phenotypes occurred after the worm's development and reproductive lifespan were completed, which was about 8–9 days of the age for the worms.

**Discussion**

APs and CYPs are increasingly detected in freshwater cyanobacterial blooms (Gkelis et al., 2015, Beversdorf et al., 2017), yet their potential toxicological effects to aquatic systems is largely unknown. Here we found that these two groups of cyanopeptides induced significant toxicity effects in a model organism the nematode *C. elegans* at 10 μg/L, a concentration that has been set as regulatory guideline for MCs in recreational waters (WHO, 2003). This concentration is also 100 times lower than those reported in the environment (Gkelis et al., 2015). At an identical mass concentration of 10 μg/L, APs were the most toxic among all the cyanopeptides tested, indicated by reduced reproduction, reduced lifespan, delayed hatching, reduced growth, and severe vulval integrity defects in the worm. APs have been found to inhibit carboxypeptidase A as well as protein phosphatases but with a ten-fold lower potency than MCs (Sano et al., 2001). A recent study reported that an AP-producing cyanobacteria strain strongly inhibited the growth of a freshwater amoeba (Urrutia-Cordero et al., 2013). MCs are among the most extensively studied cyanotoxins and are known for their hepatotoxicity (Faltermann et al., 2014), reproductive toxicity (Chen et al., 2016), potential neurotoxicity (Hu et al., 2016), and possible carcinogenicity (Zegura B, 2016). The current WHO guideline for MCs is 1 μg/L for drinking water (WHO, 2008) and 10 μg/L for recreational water (WHO, 2003). The fact that these three APs tested in the current study showed greater toxicity effects than MC-RR in the worm at the regulatory concentrations demonstrates that these cyanopeptides may pose high risks to ecosystem and human health. Our findings strongly suggest the need for more systematic understanding of the potential ecotoxicity of these cyanopeptides.

CYPs have been frequently detected in co-occurrence with MCs during blooms and are often considered as non-toxic in general (Neumann et al., 2000, Beversdorf et al., 2017). They are mainly known for their inhibition of serine proteases like chymotrypsin or trypsin. For example, CYP 1020 showed potent inhibitory activity to crustacean and mammalian serine proteases (Gademann et al., 2010). However, the uptake mechanism, molecular effects and mode of action of these cyanopeptides to fish and mammals remains elusive. A recent study by Faltermann et al. (2014) evaluated the molecular effects and mode of actions of CYP1020 in zebrafish embryos using transcriptomics. The authors found that the most clearly affected pathways were those related to DNA damage recognition and repair, circadian rhythm and response to light. This suggests that CYP 1020 may act on DNA and has neurotoxic activity. Here we found three CYPs (CYP 1007, 1020, and 1041) showed comparable toxicity to MC-RR to the worm, indicated by reduced reproduction, reduced lifespan, and increased vulval integrity defects. While the exact modes of action leading to these toxicity outcomes in the worm is unclear, our findings demonstrate that these CYPs can pose significant risks to aquatic organisms.

Microginins are linear nonribosomal cyanopeptides that are also commonly found in co-occurrence with MCs (Carneiro et al., 2012). Numerous Microginin congeners have been isolated from several cyanobacteria genera since it was first reported over two decades ago (Stewart et al., 2018). They usually contain 4-6 residues including one or more tyrosine at the C-terminus and the characteristic N-terminal side chain, 3-amino-2-hydroxy decanoic acid (Fig. 2.1). Microginins are renowned inhibitors of the angiotensin-converting enzyme (ACE) and may serve as candidates for the discovery of novel antihypertensive agents (Carneiro et al., 2012). It is generally believed that toxicity of microginins are much lower than that of MCs (Zanchett and Oliveira-Filho, 2013). Our findings seem to agree with this as we found that miroginin 690 was the least toxic among all the cyanopeptides examined, including MC-RR.

*C. elegans* serves as a good model to illustrate how chemical exposures by ingestion, of both food and water, may pose a hazard to the health of an organism from embryogenesis to death (Page and Johnstone, 2007, O'Reilly et al., 2014). The worm has a cuticle that makes absorption of toxins difficult, thus the exposure occurs primarily through ingestion (Hunt, 2017). We first examined how these cyanopeptides may affect the reproduction of the worm. We found that at a concentration as low as 10 µg/L, both APs and CYPs significantly decreased the worm's reproduction as indicated by decreased numbers of progeny (Fig. 2.2). This change in the number of progeny and the ability of eggs to take up nutrients can cause downstream effects that may affect embryogenesis and growth, and impact the overall healthspan (Gardner et al., 2013). In *C. elegans*, embryogenesis, from fertilization to egg hatching, takes approximately 12–13 h at 20 °C, and consists of both utero (2–3 h) and ex utero (9–10 h) development (Harlow et al., 2016). This makes hatching time a convenient measure to study the potential impact of chemicals on the worm's developmental processes, such as cell fate specification, tissue formation, and morphogenesis (Harlow et al., 2016). We found that both APs and CYPs significantly delayed hatching of the worm (Fig. 2.3), suggesting the impact of these cyanopeptides on the worm's developmental processes. The long-term effect of this delayed hatching to the worm's overall health is not known yet and warrants further investigations. Further research is also needed to understand how these cyanopeptides impact the embryonic development processes that leads to delayed hatching.

Following the delayed hatching, we also found that both groups of cyanopeptides reduced the worm's growth rate as indicated by reduced body length at different larval stages. Upon hatching, *C. elegans* progresses through four distinct larval stages (L1-L4) and reach adulthood at approximately 3 days (72 h) of age (Uppaluri and Brangwynne, 2015). We observed decreased body length of the worms exposed to cyanopeptides at 18, 24, 48, and 72 h (Fig. 2.4). The body size of *C. elegans* is genetically regulated and interference of gene expression in pathways involved in stage-specific development in the worm can result in "heterochronic" growth abnormalities (Zaidel-Bar et al., 2010, Monslave et al., 2011). Previous research suggests that there are at least three different genetic pathways that determine the worm's body length: 1) a spectrin pathway involving the *unc-70, sma-1*, and *spc-1*; 2) a calcineurin pathway involving *tax-6* and *cnb-1*; and 3) a transforming growth factor-b (TGF-B) pathway involving *sma-2, sma-3, sma-4*, *lon-1, kin-29*, and *dbl-1* (Morck and Pilon, 2006). Additionally, the absence of food can halt development. This, however, is not likely to be the reason for the observed growth impact in our study as the worms were fed throughout the exposure duration. Our future studies will examine the impact of these cyanotoxins on these three pathways involved in larval development to elucidate the potential mechanisms of toxicity leading to the decrease in body size. Furthermore, the long-term health implication of this decrease in body size also calls for further investigations.

Similar to reproduction and hatching time, all APs and CYPs significantly decreased the average lifespan of the worm (Fig. 2.5). Lifespan is regulated by complex interactions involving stochastic events, genetics, environment, and other nongenetic factors (Uno and Nishida, 2016). An important regulatory pathway affecting longevity, as well as healthspan and aging in the worm, is the insulin/insulin-like growth factor 1 (*IGF-1*) signaling pathway (Gao et al., 2017). IGF signaling is controlled by the activation of *daf-2* (IGFR) that initiates a signaling cascade involving *age-1* (phosphoinositide 3-kinase), *pdk-1* (phosphoinositide-dependent protein kinase-1) and *akt-1/2* (protein kinase B) (Murphy et al., 2003, Murphy, 2013). Activation of this signaling cascade results in the phosphorylation of *daf-16* and inhibits its relocation into the nucleus (Ogg et al., 1997). IGF signaling plays a vital role in prenatal growth, fertility, regulating fat storage and blood glucose, stress response, and aging in the worm (Laviola et al., 2008, Zhang and Liu, 2014). Overactivation of IGF signaling is associated with accelerated aging and increased mortality in the worm (Fadini et al., 2010). The significant reduction of lifespan, together with the reduced reproduction and growth induced by these APs and CYPs may suggest that the IGF-mediately pathway is involved in causing these effects. Our next step is to examine the impact of these cyanotoxins on the activity of the IGF signaling pathway using mutant strains and gene expressions.

A remarkable finding from our study is that APs induced severe vulval integrity defects (Avid) in adult worms during the aging process (Fig. 2.6). The Avid phenotype in aging *C. elegans* has been long observed yet only clearly described and studied by Leiser et al. (2016) recently. The researchers examined the phenotype in the context of several genetic and environmental conditions that are known to modify worm longevity and concluded that Avid is a definitive cause of healthspan loss that is associated with early death. The phenotype is easily identifiable and can therefore be a robust marker of worm healthspan. We found that the extremely high incidence of Avid in worms exposed to APs corresponded well with the most significantly shortened lifespan (reduction by 30% compared to controls) induced by these cyanopeptides. Leiser et al. (2016) suggested that the worm's reproductive system (germline) is an important modulator of Avid as they found the loss of oocyte development is associated with increased Avid. The researchers also proposed two hypotheses for this association. One is that accumulation of excess, unutilized yolk caused by loss of oocyte development leads to leakage of body fluids and organs through the vulva. Another hypothesis considers Avid as a result from accumulation of nutrients related to the worm's reproduction. Because the metabolism of food and nutrients in post-mitotic *C. elegans* is largely geared toward the production of 150–300 viable eggs per adult hermaphrodites, a decrease in the number of eggs or the ability of the eggs to absorb nutrients could lead to excess nutrients in the adult worm. In the present study, we observed that the three APs significantly reduced the worm's reproduction (number of eggs), which may potentially contribute to the development of Avid phenotype in the adult worms at a later stage.

Another interesting aspect of Avid phenotype is the potential link between this worm-specific phenotype to mammalian health. Leiser et al. (2016) suggested that Avid in the worm may be a phenotype analogous to obesity in higher organisms. Such a hypothesis is intriguing, considering that the yolk in the worm is analogous to visceral fat in mammals, which is known to lead to numerous health problems (Seidell et al., 1990). While the potential link between Avid and obesity is yet to be proven, it has the potential to connect a worm-specific phenotype directly to mammalian health, which will have great implication for our understanding of the potential health impacts of these cyanotoxins on humans. Our future studies will examine the potential link between Avid and obesity by comparing fat accumulation, fat metabolism, systemic iron overload, and ferritin levels (Hyun et al., 2016, Wang et al., 2016) in controls and worms exhibiting the avid phenotype.

**Conclusions**

The increasing detection of cyanopeptides such as APs and CYPs co-occurring with highly toxic MCs in freshwater systems calls for immediate attention to understand their potential ecological and human health impacts. To the best of our knowledge, this is the first study that has examined the toxicological effects of APs and CYPs at an environmentally and regulatorily relevant concentration (10 µg/L) to a model organism from organismal levels. We found that three APs (AP-A, AP-B, and AP-F) and three CYPs (CYP-1007, CYP-1020, and CYP-1041) induced significant toxicity effects to the worm at concentrations 100 times lower than those found in the environment. These toxicity effects include reduced reproduction, delayed hatching, decreased growth rate, shortened lifespan, as well as severe aging-related vulval integrity detects. At an identical mass concentration of 10 µg/L, APs were the most toxic, followed by CYPs, which showed comparable toxicity to MC-RR, and Microginin 690 was the least toxic. These findings demonstrate that these APs and CYPs may pose significant health risks to aquatic organisms, and more toxicological studies of these cyanopeptides using different species across different trophic levels are needed to gain a thorough understanding of their potential impact on the ecological system. Future studies should also aim to understand whether these observed toxicity in the organismal level in the worm can be related to the known protease inhibition properties of these cyanopeptides. Given the frequent co-occurrence of these APs and CYPs with MCs as well as their comparable or even greater toxicity than MCs, potential recreational and drinking water guidelines should consider taking into account these cyanopeptides as well.

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CHAPTER 3:

Triclosan (TCS) and triclocarban (TCC) induce systemic toxic effects in a model organism the nematode Caenorhabditis elegans


**Abstract**

The broad application of triclosan (TCS) and triclocarban (TCC) as antimicrobials in household and personal care products has led to the concerns regarding their human health risk and environmental impact. Although many studies have examined the toxicological effects of these compounds to a wide range of aquatic organisms from algae to fish, their potential toxicity to an important model organism the nematode *Caenorhabditis elegans* has never been systematically investigated. Here we assessed the toxicological effects of TCS and TCC in *C. elegans* using endpoints from organismal to molecular levels, including lethality, reproduction, lifespan, hatching, germline toxicity, and oxidative stress. L4 stage or young adult worms were exposed to TCS or TCC and examined using above-mentioned endpoints. Both TCS and TCC showed acute toxicity to *C. elegans*, with 24-h LC50s of 3.65 (95% CI: 3.15, 4.3) mg/L and 0.91 (95% CI: 0.47, 1.53) mg/L, respectively. TCS at 0.1–2 mg/L and TCC at 0.01–0.5 mg/L, respectively, induced concentration dependent reduction in the worm's reproduction, lifespan, and delay in hatching. Using a DAF-16:GFP transgenic strain, we found both compounds induced oxidative stress in the worm, indicated by the relocalization of DAF-16:GFP from cytoplasm to the nucleus upon exposure. Germline toxicity of the two compounds was also demonstrated using a xol-1:GFP transgenic strain. These findings suggest that TCS and TCC induce systemic toxic effects in *C. elegans*. Further studies are needed to elucidate the potential mechanisms of toxicity of these antimicrobials in the model organism, especially their potential endocrine disruption effects.

## Introduction

Triclosan (TCS) and triclocarban (TCC) are broad-spectrum antimicrobial agents used in numerous personal care, veterinary, industrial and household products (Dann and Hontela, 2011). The two main sources of TCS and TCC release into the environment are discharge of wastewater effluent into surface waters and land application of biosolids containing residues of the antimicrobials (Dhillon et al., 2015). Human exposure to TCS and TCC through direct contact with daily consumer products or consumption of contaminated water and food has led to their detection in human milk, plasma, and urine (Dhillon et al., 2015). TCS and TCC are among the top 10 most commonly detected organic wastewater compounds in terms of frequency and concentration (Halden and Paull, 2005). Literature shows that concentrations of these antimicrobials in natural aquatic environment typically range from below the detection limit to maxima of 2.3 μg/L for TCS and 0.25 μg/L for TCC in U.S. surface waters (Chalew and Halden, 2009). TCS concentrations in freshwater sediments range from 800 to 53,000 μg/kg, and TCC in estuarine sediments range from 1700–24,000 μg/kg (Chalew and Halden, 2009).

The main heath concerns to the ecosystem and humans induced by TCS and TCC contamination in the environment include antibiotic resistance, ecotoxicity, and potential endocrine-disrupting effects (Higgins et al., 2011). As microbial resistance has become an increasingly serious public health problem, the continued use of antimicrobials such as TCS and TCC may exacerbate this problem. Although these antimicrobials are not acutely toxic or carcinogenic in mammalian toxicity studies, their structural similarity with estrogenic or androgenic endocrine-disrupting compounds (EDCs) (such as PCBs, PBDEs, BPA, and dioxins) leads to the suspect that they may act as endocrine disruptors (Haggard et al., 2016). Many studies have shown that TCS has the ability to disrupt endocrine function in a variety of species including fish, amphibian, and mammals (Dann and Hontela, 2011). These studies have collectively shown that TCS has estrogenic and/or androgenic activity at environmentally relevant concentrations. For example, TCS exposure has been found to induce changes in fin length and sex ratios of medaka fish, demonstrating a weak androgenic effect (Foran et al., 2000). A later study by Ishibashi et al. (2004) found that TCS induced vitellogenin production in male medaka, suggesting estrogenic activity. Studies on the endocrine disruption potential of TCC are scarce compared to TCS. Several researchers have reported that TCC showed no endocrine activity when tested alone but enhanced estrogenic or androgenic activities following co-exposure with estrogen or dihydrotestosterone, potentially representing a new type of endocrine disruptor (Chen et al., 2008, Tarnow et al., 2013).

For ecotoxicity, acute toxicity of TCS and TCC has been studied in invertebrates, fish, amphibians, algae, and plants (Brausch and Rand, 2011). Studies on TCC have been much limited as compared to TCS, although TCC is usually found more toxic than TCS to aquatic invertebrates and fish for both short and long-term exposures (Chalew and Halden, 2009). Different environmental species exhibit great variations in their susceptibility to TCS and TCC, with toxicity threshold values spanning more than six orders of magnitude in concentration. Threshold concentrations for acute toxicity in fish were between 260 and 440 μg/L for TCS and 49–180 μg/L for TCC, and for chronic effects were 34–290 μg/L for TCS and 5 μg/L for TCC (Ishibashi et al., 2004, Tatarazako et al., 2004). In crustacean, acute toxicity threshold values ranged 185–390 μg/L for TCS and 1.9–40 μg/L for TCC, and chronic toxicity was observed at concentrations as low as 6–182 μg/L for TCS and 0.06–4.7 μg/L for TCC (Tatarazako et al., 2004). Algae appears to be the most sensitive species to TCS and TCC toxicity, with toxicity effects observed at parts-per-billion levels ranging 0.2–2.8 μg/L for TCS and 10–30 μg/L for TCC (Brausch and Rand, 2011). The high sensitivity of algae to TCS toxicity is likely due to the similarity in lipid synthesis process between algae and bacteria, which is the target for TCS antibacterial properties. The potential mechanisms of toxicity for TCS to aquatic organisms have not been well defined, but may include non-specific narcosis in some species and specific actions in others (Lyndall et al., 2010). These specific mechanisms include inhibition of the type II fatty acid synthesis enzyme system, membrane destabilization, and uncoupling of oxidative phosphorylation (Franz et al., 2008). Unfortunately, the mechanism of toxicity for TCC remains largely unknown.

In contrast to the abundant literature regarding TCS and TCC toxicity on aquatic biota, studies of their toxicity to terrestrial organisms are limited. TCS has been reported to inhibit plant growth and soil respiration, with EC50s ranging 57–108 mg/kg (Liu et al., 2009). TCS has also been found to disturb nitrogen cycle in some soils at concentrations below 10 mg/kg (Waller and Kookana, 2009). An ecological risk assessment of TCS in terrestrial environment performed by Reiss et al. (2009) found satisfactory margins of safety for terrestrial organisms including earthworms, plants, and soil microorganisms exposed to TCS in soils amended with sewage sludge, and birds and mammals exposed indirectly through the consumption of earthworms and fish. However, the small number of studies available for the risk assessment calls for further investigations of the potential impact of TCS and TCC on terrestrial organisms.

*C. elegans* is a free-living nematode in soil environments, yet its natural habitat is comparable to the habitat of freshwater nematodes (Hoss and Weltje, 2007), making it suitable for chemical exposures in both soil and aqueous medium. It is a representative species in the nematode phylum with great ecological relevance. It is also an excellent model organism for toxicological studies due to its well-understood biology, genetic amenability, high conservation with human biology and disease pathways, and ease of culture in the laboratory (Leung et al., 2008). However, there has been no systematical studies on toxicological effects of TCS and TCC to this nematode species except one with inconclusive findings on the toxicity of these compounds on *C. elegans* growth and fecundity (Inokuchi et al., 2014). Here we investigated the toxicological effects of TCS and TCC to *C. elegans* using a series of endpoints from organismal to molecular levels. Overt endpoints including lethality, reproduction, lifespan, and hatching time were examined to gain a basic understanding of their general toxicity to this model organism. Transgenic strains targeting oxidative stress response and germline toxicity were then used to assess toxicity at molecular levels. Findings from this study are expected to help fill the knowledge gap regarding the toxic effects of these antimicrobials to an ecologically relevant model organism. They will also provide the foundation for future mechanistic investigations in this model organism to further understand the environmental and human health implications of these antimicrobial chemicals.

## Materials and methods

### Chemicals

Triclosan and triclocarban were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (1000 mg/L) were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO). All chemical concentrations used for sub-lethal exposure studies were selected based on lethal concentrations (LC50s). All dilutions used K-medium (51 mM NaCl, 32 mM KCl). TCS exposure concentrations were 0.1–5 mg/L, and TCC exposure concentrations were 0.01–5 mg/L. The final DMSO concentration in working solutions was <0.5%.

### *C. elegans* strains

All *C. elegans* strains used in this study were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). These include N2 Bristol (wildtype), TJ356 (daf-16p:daf-16a/b:GFP), and TY2431 (xol-1:GFP). All strains were maintained at 20 °C on nematode growth medium (NGM) agar plates seeded with *E. coli* OP50 following standard protocols (Stiernagle, 2006). Eggs were collected by bleaching gravid adult *C. elegans* with a bleaching solution of 1% NaClO and 0.013M NaOH to generate age-synchronized worm populations for all experiments described thereafter (Donkin and Williams, 1995).

**Lethality**

To assay lethality, age synchronized young adult (3-day old) nematodes were placed in 24-well plates containing 1 mL of K-medium (control), TCS (1–5 mg/L), or TCC (0.05–5 mg/L) solutions. Each test concentration contained three wells and each well contained ten worms. The exposure lasted for 24 h and the worms were not fed during the exposure. Following 24-h exposure, mortality of the worms was examined under a dissecting microscope. LC50s were calculated using logistic regression in the SigmaPlot program (Systat Software, Inc, San Jose, CA). The experiment was independently repeated three times for lethality test and the tests described thereafter unless stated otherwise.

**Reproduction and lifespan**

Reproduction tests were performed using age synchronized L4-stage nematodes on OP50 seeded NGM plates. Because TCS or TCC inhibits the growth of OP50, they were not added into NGM media or mixed with OP50 broth; instead, an aliquot (150 μL) of the chemical solution was added on the surface of OP50 plates and was spread evenly using a sterilized glass rod. This approach for introducing chemicals to the exposure system was also used in the subsequent experiments on lifespan, hatching time, and germline toxicity. The concentrations used for TCS were 0.1, 1, and 2 mg/L, and for TCC were 0.01, 0.1, and 0.5 mg/L. One L4-stage worm was added to each plate and each exposure concentration contained six plates. The exposure was conducted at 20 °C and continued from L4 stage to the end of reproduction period (approximately 4–6 days in total). For each plate, following the start of exposure, the parent worm was transferred to a fresh exposure plate every other day and the number of progeny (eggs and larval worms) in the original plate was counted. At the end of exposure, the number of progeny from all plates were added for each exposure concentration, and the average number of progeny was calculated.

Lifespan experiment followed the protocol as described previously (Pluskota et al., 2009). Briefly, L4 stage worms were placed on NGM plates seeded with OP50, which has been amended with 0.2 mM 5-fluoro 2-deoxyuridine (FUdR) to prohibit progeny production during lifespan experiment. Similar to the reproduction experiments, 150 µL of K-medium (control), TCS (0.1, 0.5, 1, and 2 mg/L), or TCC (0.01, 0.05, 0.1, and 0.5 mg/L) were spread evenly over the surface of the plates. 100 worms were placed on each plate and each exposure concentration contained three plates (300 worms for each concentration). Plates were kept at 20 °C and the number of worms that were alive was recorded each day. Worms were considered dead when they failed to respond to external stimuli by gentle tapping of the worm's head with a platinum transfer picker. Dead worms were removed from the plate. Worms that crawled off the plates or burrowed under the agar were excluded from the population. This process was repeated until all the worms died. Log-rank survival analysis was applied to life-span analysis using SPSS 23 software (IBM SPSS Statistics, Armonk, NY).

**Hatching time**

Hatching time is defined as the time from egg deposition to hatch (Muschiol et al., 2009). To assay hatching time, eggs from age-synchronized 3-day nematodes were collected as previously described (Donkin and Williams, 1995). Eggs were centrifuged and washed with K-medium twice, and were placed on OP50 plates amended with 150 µL of K-medium (control), TCS (0.1 0.5, 1, and 2 mg/L), or TCC (0.01, 0.05, 0.1, and 0.5 mg/L). Approximately 100 eggs were placed on each plate and each exposure concentration contained three plates. The number of eggs hatched were recorded every hour until all eggs hatched.

### Stress response

A transgenic strain, TJ356 [daf-16p:daf-16a/b:GFP + rol-6], which stably expresses a DAF-16:GFP fusion protein, was used to assess the stress response in the worm induced by TCS or TCC exposure. DAF-16 is the sole *C. elegans* homologue of the Forkhead-box Class O (FOXO) transcription factor in mammals, and its activity is regulated by cellular stress signals especially oxidative stress (Hesp et al., 2015). Under normal growth conditions, DAF-16 is inactivated and is primarily located in the cytoplasm; when activated by oxidative or thermal stress, it is translocalized to the nucleus where it regulates gene expressions including antioxidant genes, chaperones, and metabolic genes to increase stress tolerance (Baumeister et al., 2006). Such DAF-16:GFP relocalization can be visualized by fluorescence microscopy. To test DAF-16 relocalization, L4-stage nematodes were exposed to 1 mL of K-medium (control), TCS (0.1 and 1 mg/L), or TCC (0.01 and 0.1 mg/L) in a 24-well plate for 4 h and were transferred to fresh NGM plates to recover for 30 min. A heat-shock treatment (4-h exposure at 34 °C) was used as positive control. Upon recovery, worms were placed on agar pad on glass slides and immobilized with 0.1% tricaine and 0.5% levamisole. Worms were imaged on a Zeiss Axio ZV16 fluorescence microscope (Carl Zeiss Microscopy, LLC. Thornwood, NY). Sixty worms were imaged for each exposure concentration and the frequency of GFP relocalization was calculated.

### Germline toxicity

Germline toxicity test was conducted using a xol:GFP (TY2431) transgenic strain. The principle of using this transgenic strain to assess germline toxicity has been described in detail by Allard et al. (2013). The worm has a rare proportion of male progeny (XO, <0.2%) that naturally occurs in the wild-type hermaphroditic (XX) population due to a meiotic segregation error of the X-chromosome (Hodgkin et al., 1979). Disruption of meiosis (germline toxicity) frequently leads to increased aneuploidy and errors in X-chromosome, which usually correlates with a “high incidence of males” phenotype (Allard et al., 2013).

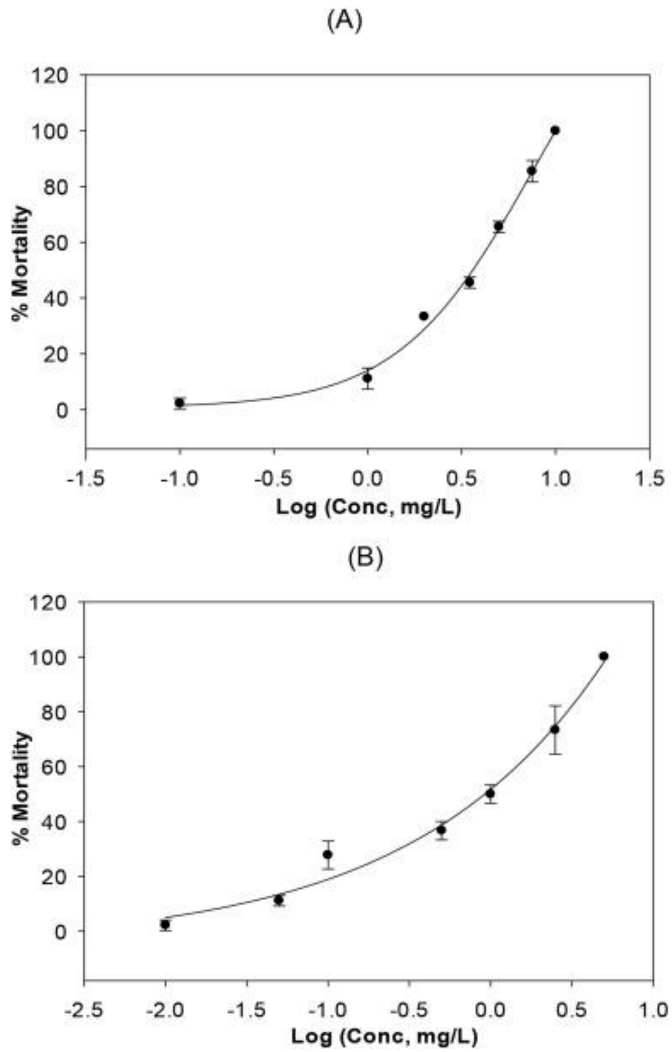
The xol-1:GFP transgenic strain uses a male-specific promotor (xol-1) to drive expression of GFP, which allows a quick identification of male embryos by the appearance of “green eggs” within the worm's hermaphrodite uterus. The appearance of “green eggs” can thus be used as an indicator of toxicity to the *C. elegans* germline system.

To assay germline toxicity, L4-stage worms were placed on OP50 plates with 150 µL of K-medium (control), TCS (0.1 and 1 mg/L), or TCC (0.01 and 0.1 mg/L) evenly spread over the surface. The exposure lasted for 24 h. Upon exposure, worms were removed from the exposure plates and washed with K-medium twice. The worms were then examined under fluorescent microscope using the method described in the previous section for appearance of GFP-positive eggs. Fifty worms were examined for each exposure concentration and the experiment was repeated three times. A total of 150 worms were observed for each exposure concentration or control. The frequency of “green eggs” phenotype was calculated. The number of “green eggs” were also counted and recorded. Nocodazole, a known antimitotic agent in mammalian model, was used as positive control (Allard et al., 2013). A concentration of 100 µM nocodazole was used in the experiment.

**Data analysis**

All experiments were independently repeated for three times. LC50s were calculated using Sigmaplot software (Systat Software Inc. USA). Comparison of differences among multiple groups of exposure conditions was performed by one-way analysis of variance (ANOVA). Lifespan was analyzed using Kaplan Meier survival analysis to estimate the mean lifespan (IBM SPSS Statistics, Armonk, NY). The log-rank test was used to compare the survival function between the control and exposure groups.

Figure 3.1. Acute lethal toxicity of triclosan (TCS) and triclocarban (TCC) to *C. elegans*.




(A) TCS, 24-h LC50 = 3.65 mg/L (95% CI: 3.15, 4.3); (B) TCC, 24-h LC50 = 0.91 mg/L (95% CI: 0.47, 1.53). Error bars indicate standard errors of the means based on three independent replicate experiments (n = 3).

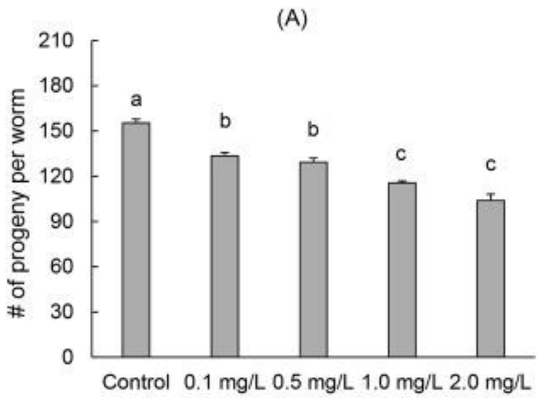
**Results and discussion**

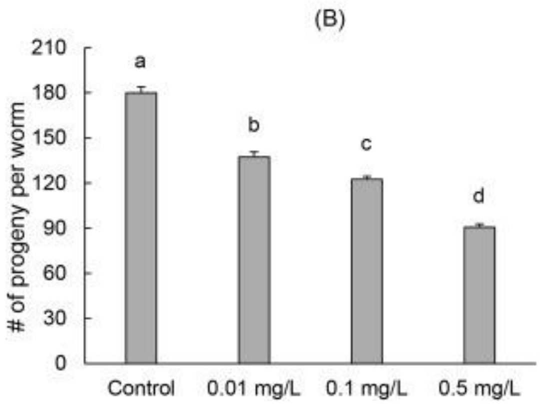
**Effects of TCS and TCC on *C. elegans* mortality, reproduction, hatching, and lifespan**

We found both TCS and TCC induced acute lethal toxicity to *C. elegans*. 24-h LC50s for the two compounds were 3.65 (95% CI: 3.15, 4.3) and 0.91 (95% CI: 0.47, 1.53) mg/L, respectively (Fig. 3.1). To the best of our knowledge, this is the first report of the acute lethal toxicity of these two antimicrobials in this model organism. These acute LC50 values are in general one order of magnitude greater than those found in fish and aquatic invertebrates (Dann and Hontela, 2011), indicating that *C. elegans* may not be as sensitive to TCS and TCC toxicity as those aquatic species. Toxicity data of TCS and TCC on terrestrial species have been very limited. Several recent studies have examined TCS toxicity on the earthworm (*Eisenia fetida*). Using filter paper contact test, the authors reported 48-h LC50s of TCS ranging 0.004–0.008 $mg/cm^2$ (Gillis et al., 2017). Because the filter paper test does not translate well into effective concentrations in liquid medium, it is difficult to compare the TCS toxicity between *C. elegans* and earthworms. Another study found that the 14-d LC50 of TCS to the earthworm was greater than the highest concentration tested, 1026 mg/kg soil dry weight (V. Wuthrich, Report 262956, RCC, Umweltchemie, Itingen, Switzerland, unpublished data). Based on a comprehensive review on existing toxicity data of triclosan to a broad range of terrestrial organisms, Reiss et al. (2009) concluded that the sensitivity of terrestrial organisms to triclosan is substantially lower than the sensitivity of aquatic species. Our findings seem to agree with this statement. We found TCC was more toxic than TCS to the nematode, indicated by its significantly lower LC50 values, which is consistent with previous findings on aquatic invertebrates and fish (Brausch and Rand, 2011).

Both TCS and TCC caused concentration dependent impact on *C. elegans* reproduction at concentrations one to two orders of magnitude lower than those causing acute lethality toxicity. For TCS, concentrations of 0.1 and 2 mg/L caused reduction of the worm brood size from 153 (±11) in the

Figure 3.2. Impact of TCS or TCC exposure on *C. elegans* reproduction.



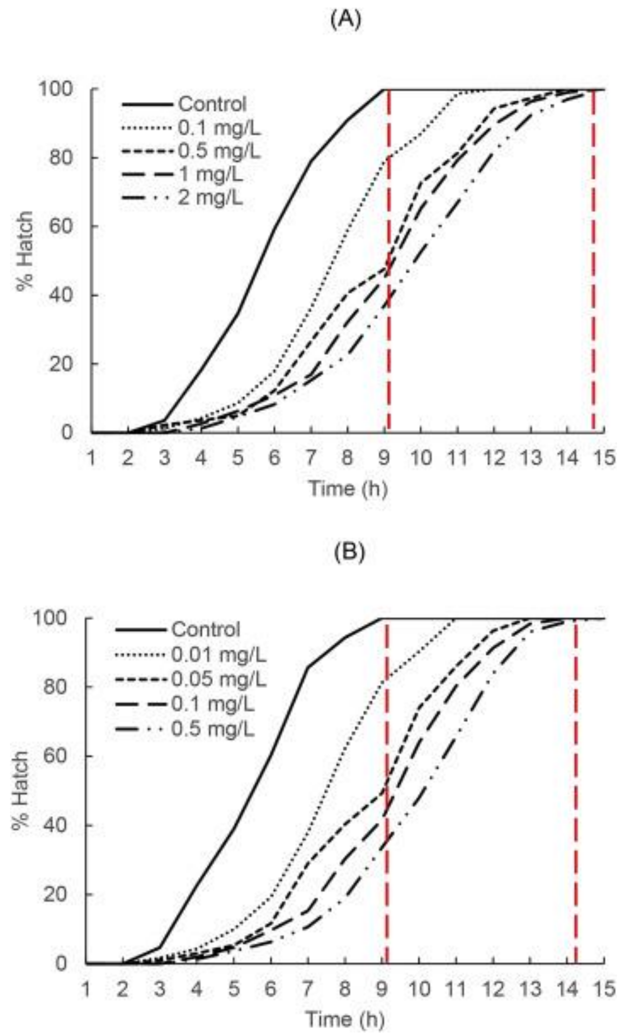




(A) TCS, (B) TCC. Error bars indicate standard errors of the mean based on three independent replicate experiments (n = 3). Different letters indicate significant differences according to Tukey's HSD test in conjunction with ANOVA analysis ($p < 0.05$).

control to 132 (±9) and 102 (±12), respectively, representing a 13.7% and a 33.9% reduction from the control (Fig. 3.2A). TCC showed similar effect on *C. elegans* reproduction, but at much lower concentrations. At concentrations as low as 0.01 mg/L, TCC induced a 23.7% decrease in brood size from the control (Fig. 3.2B). These findings were different from those reported by Inokuchi et al. (2014), where the estimated LOECs (lowest observed effect concentration) to *C. elegans* reproduction were 0.11 mg/L for TCC and >6.25 mg/L for TCS. Unfortunately, as the experimental method as well as the data were not described in sufficient detail in the Inokuchi et al. (2014) study, possible reasons for such discrepancy in observed reproduction toxicity could not be deduced. Similar effects of these antimicrobials on reproductive system have been reported in a marine invertebrate the monogonont rotifer (*Brachionus koreanus*) (Han et al., 2016). The researchers reported LC50s of 0.393 mg/L for TCS and 0.388 mg/L for TCC in the rotifer, and both compounds at 0.2 mg/L reduced the fecundity of the organism. Impact of these compounds on the reproduction of environmental organisms may have a deleterious effect on their lifecycle and potentially affect the ecosystem. While most of the existing literature on toxicity effects of these antimicrobial compounds (including those used in risk assessment) are based on acute lethal toxicity, more studies are needed to understand their potential impact on sublethal effects such as reproduction to improve the hazard and risk assessment of these compounds.

TCS and TCC also affected *C. elegans* egg hatching. TCS at concentrations ranging 0.1–2 mg/L delayed *C. elegans* hatching at a concentration-dependent manner (Fig. 3.3A). For the control, all eggs hatched by 9 h under 20 °C; at the lowest concentration of 0.1 mg/L of TCS, all eggs hatched by 11 h, with a 2 h delay in hatching. For the highest TCS of 2 mg/L, hatching was delayed by 5.7 h. Similar results were observed for TCC. At the lowest concentration of 0.01 mg/L, hatching was delayed by 2 h; and at the highest concentration of 0.5 mg/L, hatching was delayed by 5 h (Fig. 3B). Interestingly, although significant delay in hatching was observed, all hatched larvae developed into normal adults within 2–3 days. *C. elegans* development and the underlying genetic mechanisms controlling it have been well

Figure 3.3. *C. elegans* hatching time under different TCS or TCC exposure.

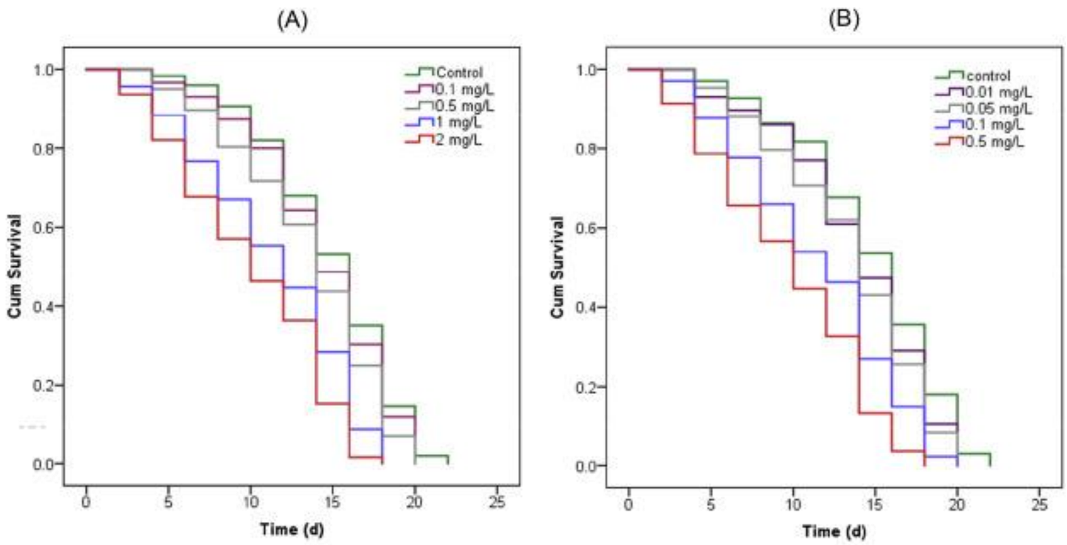



(A) TCS, (B) TCC. Under control conditions, all worm eggs hatched by 9 h; 0.1–2 mg/L TCS caused a hatching delay by 2–5.7 h and 0.01–0.5 mg/L TCC induced a hatching delay by 2–5 h. 300 worm eggs were tracked for hatching at each exposure concentration.

described (Priess, 2005). Embryogenesis, from fertilization to egg hatching, takes approximately 13–14 h at 20 °C, during which numerous developmental processes occur, leading to cell fate specification, tissue formation, and morphogenesis (Harlow et al., 2016). The embryogenesis consists of *in utero* development (~150 min at 22 °C) and *ex utero* development (9 h at 22 °C), with the later referring to from egg laying to hatching (Altun and Hall, 2006). Therefore, scoring hatching time (from eggs laid to hatching) is a convenient way to assess the success of these developmental processes. We observed delayed hatching of the embryonic *C. elegans,* suggesting that developmental processes were impacted by TCS or TCC exposure. TCS and TCC have been reported to affect the development of embryonic zebrafish. Oliveira et al. (2009) reported a delayed hatching of zebrafish embryos accompanied by spine malformations and pericardial edema upon exposure to 0.5 mg/L TCS, and exposure to 0.9 mg/L of TCS caused considerable delay on the otolith formation and body pigmentation. Although all larval worms from delayed hatching developed into normal adults, any delayed or transgenerational effects from the hatching delay warrant further investigation. Our future study will track the worms from delayed hatching to the next two generations by examining the fecundity and development to investigate the potential transgenerational effects.

Similar to reproduction, both TCS and TCC reduced *C. elegans* lifespan in a concentration dependent manner. At concentrations ranging 0.5–2 mg/L, TCS reduced the average lifespan by 1.3–4.8 days (Fig. 3.4A). Similarly, TCC at concentrations of 0.05–0.5 mg/L caused a lifespan reduction by 1.3–5 days (Fig. 3.4B). Animal lifespan is a result of complex interactions between genetic, environmental, and other stochastic factors (Uno and Nishida, 2016) and can provide valuable insights into the entire life cycle affected by environmental chemicals. *C. elegans* lifespan has been widely used as an endpoint for chemical toxicity testing including heavy metals and mycotoxins (Wang et al., 2010, Yang et al., 2015), although the exact mechanism(s) regarding how chemicals impact the worm's lifespan remains unknown.

Figure 3.4. Impact of TCS or TCC exposure on *C. elegans* lifespan.




(C)

| TCS (mg/L) | Lifespan (d, 95% CI) | TCC (mg/L) | Lifespan (d, 95% CI) |
|---|---|---|---|
| Control | 14.8 (14.3, 15.2) | Control | 14.7 (14.2, 15.2) |
| 0.1 | 14.2 (13.8, 14.7) | 0.01 | 13.8 (13.4, 14.4) |
| 0.5 | *13.5 (13.0, 13.9) | 0.05 | *13.4 (12.9, 13.9) |
| 1 | *11.3 (10.8, 11.8) | 0.1 | *11.5 (10.9, 12.0) |
| 2 | *10.0 (9.5, 10.5) | 0.5 | *9.7 (9.2, 10.3) |

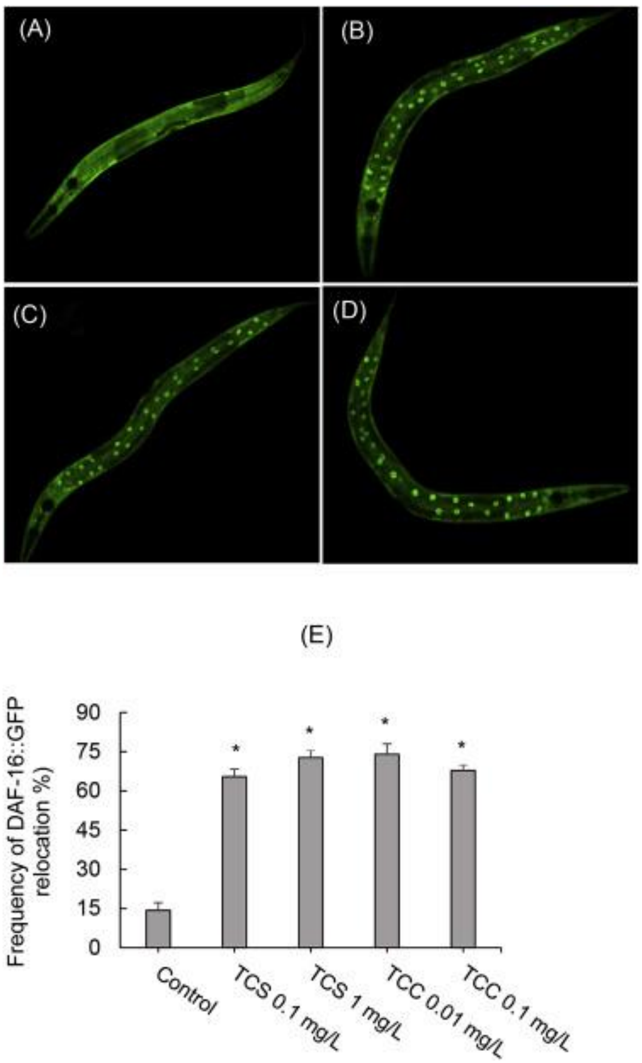
(A) TCS, (B) TCC, (C) mean lifespan under different exposure conditions from Kaplan Meier survival analysis. "*" denotes significant differences from control mean lifespan based on log-rank test ($p < 0.001$). 300 worms were tracked for survival at each exposure concentration.

An important regulatory pathway affecting aging and longevity in *C. elegans* is the signaling pathway through the insulin/insulin-like growth factor 1 (IGF-1) receptor, one of the best-characterized genetic regulatory networks in the worm. Modulation of insulin/IGF-1 signaling in *C. elegans* is the central determinant of the endocrine control of stress response and aging (Baumeister et al., 2006). The signaling pathway contains two key upstream components, DAF-2 and AGE-1, and three important downstream regulatory transcription factors, DAF-16/FOXO, HSF-1 (heat shock transcription factor 1), and SKN-1 in regulating aging and lifespan (Altintas et al., 2016). The pathway signals through a series of well-organized sequential events, depending on environmental conditions. Under favorable conditions, the insulin/IGF-1 pathway is activated and the worm undergoes normal development and lifespan; under unfavorable conditions, the insulin/IGF-1 pathway is down-regulated and leads to the activation of DAF-16/FOXO through promoting its translocation from the cytoplasm to the nucleus, where it turns on the expression of genes that promote longevity (Altintas et al., 2016). We observed that exposure to TCS or TCC led to reduced lifespan together with activation of DAF-16/FOXO (discussed in detail in the following section), which seems to be contradictory to what has been described for the insulin/IGF-1 pathway. It remains elusive whether the insulin/IGF-1 signaling pathway is involved in the impact of TCS or TCC on *C. elegans* lifespan, or the observed impact on lifespan and oxidative stress response are caused by completely independent mechanisms. To help elucidate this, we are currently examining the impact of these antimicrobials on the worm's lifespan in certain mutant strains targeting several key upstream or downstream components of the regulatory pathway including *daf-2, age-1, daf-16,* and *skn-1.*

**Impact of TCS and TCC on *C. elegans* stress response**

*C. elegans* is a powerful model for the study of stress response as the worm has conserved stress-response pathways mediated by DAF-16/FOXO with vertebrates including mammals (Hesp et al.,

Figure 3.5. Impact of TCS or TCC exposure C. elegans oxidative stress response in the DAF-16:GFP transgenic strain.




(A) control worm, (B) positive control - heat shock treatment, (C) worm exposed to 0.1 mg/L TCS, (D) worm exposed to 0.01 mg/L TCC, (E) frequency of DAF-16:GFP relocalization from cytoplasm to the nucleus in control or exposed worms. 60 worms were examined under each exposure concentration. Error bars indicate standard errors of the means from three independent experiments (n = 3). "*" indicates significant difference from the control ($p < 0.05$).

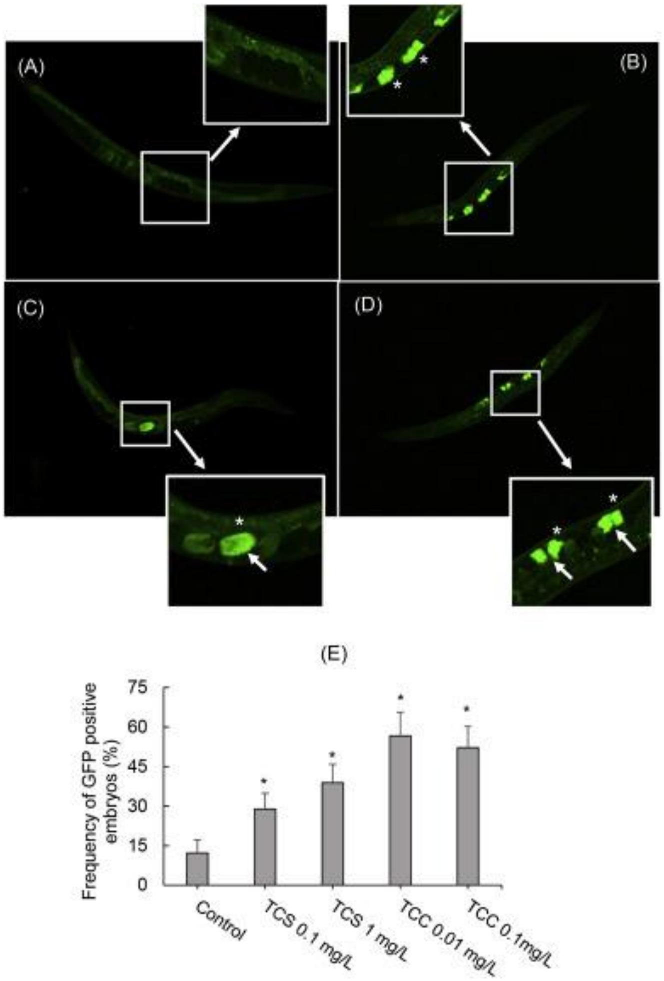
2015). The DAF-16:GFP transgenic strain has been successfully used for assessing impact of environmental chemicals including engineered nanoparticles on *C. elegans* stress response (Mohan et al., 2010). We observed clear DAF-16:GFP relocalization from cytoplasm to nucleus within the worms exposed to TCS or TCC. As shown in Fig. 3.5(A–D), DAF-16:GFP was primarily localized in cytoplasm in the control worms, but was relocalized to the nuclei of cells under heat shock treatment or TCS/TCC exposure. The frequency of such DAF-16:GFP relocalization was greater than 95% in heat shock treated worms, but was less than 15% in control worms. TCS at 0.1 and 1 mg/L induced DAF-16:GFP relocalization in 65.4 (±12)% and 72.7 (±15)% of the worms examined, respectively (Fig. 3.5E). TCC at concentrations of 0.01 and 0.1 mg/L induced such redistribution in 74 (±16)% and 67.8 (±11)% of the worms examined, respectively (Fig. 3.5E). These findings indicate that both TCS and TCC induced stress response in the worms.

This stress response may be linked to the observed toxicity effects such as reduced reproduction and lifespan induced by these antimicrobials. TCS and TCC have been reported to cause lifespan reduction and reproductive impairment through oxidative stress (generation of reactive oxygen species, ROS) and transcriptional regulation of detoxification, antioxidant, and heat shock proteins in a monogonot rotifer (Han et al., 2016). The authors found growth retardation and reduced fecundity in the rotifer in parallel with time-dependent increases in ROS production and GST enzymatic activity upon exposure to TCS or TCC. In addition, transcriptional levels of detoxification proteins (e.g., CYPs), antioxidant protiens (e.g., GST, SOD, CAT), and heat shock proteins were modulated in response to TCS or TCC exposure. Impact of TCS on fecundity, antioxidant system, and oxidative stress-mediated gene expression has also been reported in a marine copepod (Park et al., 2017). In this study, we found reduced lifespan and reproduction in parallel with increased stress response indicated by DAF-16:GFP relocalization. In *C. elegans,* DAF-16 is a major downstream target of the worm's insulin/IGF-1 pathway as well as a master transcriptional factor regulating the transcription of a broad range of genes involved in stress response, dauer formation, and longevity (Hesp et al., 2015). Our next step is to elucidate what are the downstream genes affected by DAF-16 activation in TCS or TCC exposed worms. Murphy et al. (2003) suggested that DAF-16/FOXO, once translocalized to the nucleus, upregulates genes involved in stress response and cell survival and downregulates developmental genes. The authors also found that many DAF-16 target genes encoded proteins predicted to protect cells from oxidative and other types of stress. Our future studies will examine expression patterns of the potential target genes of the DAF-16/FOXO pathway, including those involved in antioxidant system such as *sod-3, gst-4, ctl-1, ctl-4,* and those involved vitellogenesis (reproduction) such as *vit-2, vit-5*, etc., in TCS or TCC exposed worms, to gain a mechanistic understanding of the impact of these compounds on *C. elegans* stress response.

**Germline toxicity of TCS and TCC**

We employed a xol:GFP transgenic strain to assess germline toxicity induced by TCS or TCC in the nematode. Following a 24-h exposure, we observed $GFP^+$ embryos in TCS or TCC exposed worms (Fig. 3.6(A–D)). The occurrence of $GFP^+$ embryos was less than 15% in the control worms examined, and within the very few control worms that showed $GFP^+$ embryos, the number of "green eggs" within each worm was less than 2. In contrast, the appearance of $GFP^+$ embryos occurred in 35 (±12)% and 55 (±10)% of the worms exposed to 1 mg/L TCS and 0.1 mg/L TCC, respectively (Fig. 6E). Furthermore, the number of $GFP^+$ embryos within those TCS or TCC exposed worms ranged between 2 and 8, significantly greater than those in control worms. Worms exposed to 100 μM nocodazole (positive control) also showed significantly higher incidence (40%) of $GFP^+$ embryos than the control worms (12%) (Fig. 3.6B). Another notable observation was that in negative control worms and TCS (0.1 or 1 mg/L) exposed worms, the embryos within worm uterus showed normal oval shape; whereas in the positive control (nocodazole treatment) and TCC exposed worms, GFP positive embryos showed abnormal shape, suggesting reduced viability of the embryos under these exposure conditions. This is not surprising given

Figure 3.6. Impact of TCS or TCC exposure on *C. elegans* germline toxicity in the *xol-1*:GFP transgenic strain.




(A) negative control, (B) positive control - worm exposed to 100 µM nocodazole, (C) worm exposed to 1 mg/L TCS, (D) worms exposed to 0.1 mg/L TCC, (E) frequency of appearance of GFP positive male embryos in worm uterus. 50 worms were examined under each exposure concentration. Error bars indicate standard errors of the means from three independent experiments (n=3). "*" indicates significant difference from the control ($p < 0.05$). GFP positive embryos were marked with asterisk.

that nocodazole is a known antimitotic agent and a correlation between increased GFP + embryos with decreased embryo viability in nocodazole treated worms has been reported previously (Allard et al., 2013). Furthermore, decreased embryo viability in TCC exposed worms is consistent with its greater toxicity on the worm's reproduction as compared to TCS.

Both TCS and TCC induced significant germline toxicity to *C. elegans*, and TCC showed greater toxicity potency than TCS as the effects were seen at much lower concentrations. *C. elegans* germline development consists of three phases: specification, growth, and maintenance. Corresponding to life stage, germline specification occurs during the embryogenesis and early L1 stage; germline proliferation and differentiation occur during L1-L3 stage; and germline proliferation and gametogenesis occur during L4 and young adult stages – spermatogenesis occurs during the L4 stage and oogenesis during the adult stage (Hubbard and Greenstein, 2005). We started the exposure at L4 stage and the exposure continued for 24 h; therefore, effects observed from such exposure conditions capture aneuploidies originating from the disruption of any mitotic and meiotic events during germline proliferation and gametogenesis. Allard et al. (2013) has shown that a *C. elegans* screening strategy based on this transgenic strain is predictive of mammalian reproduction toxicity with an accuracy approaching 70% after examining and assessing data from over 47 compounds. The authors demonstrate that such a screening strategy can provide a quick and reliable tool to elucidate the impact of environmental chemicals on germline function in this model organism, and potentially predict mammalian reproductive toxicity. Findings from the current study are the first report that TCS and TCC induce germline toxicity in *C. elegans.* This germline toxicity agrees well with the decreased brood size induced by these antimicrobials in the worm.

Whether this germline toxicity together with the impact on reproduction and lifespan indicates potential endocrine disruption effects of TCS or TCC in the worm remains an open question. A key environmental concern of TCS and TCC is their potential endocrine disrupting effects. The majority of existing *in vivo* studies suggest that TCS has estrogenic activity although there are conflicting *in vitro* studies regarding its estrogenic and anti-estrogenic activity (Haggard et al., 2016). TCC, on the other hand, has been reported to enhance endogenous hormone activity such as testosterone (Chen et al., 2008). However, the exact mechanisms of these observed endocrine effects induced by these antimicrobials remain largely unknown. *C. elegans* provides an excellent model system for research on and assessment of endocrine disruption by environmental chemicals. The comprehensive knowledge of the genome of the worm allows for molecular, genetic, and biochemical analyses for endocrine signaling pathways. A combination of tests from different organizational levels, such as genome, proteome, organ, organism and population level, is necessary to assess endocrine disruption in order to understand its potential modes of action and ecological relevance (Hoss and Weltje, 2007). Therefore, responses from both genomic and organismal levels upon exposure to TCS or TCC in the worm are needed to understand these antimicrobials' endocrine disruption effects. We have identified effects from molecular level (oxidative stress and germline toxicity) and organismal/population level (reproduction and lifespan) in the worm which may be related to endocrine disruption by these antimicrobials. Future studies should focus on genomic and proteomic responses to these compounds in the worm with an aim to gain a complete understanding of their potential endocrine disruption effects and related ecological significance.

**Conclusion**

TCS and TCC induced significant toxic effects to a model organism the nematode *C. elegans* from apical endpoints to molecular level responses. Lethal toxicity occurred at mg/L levels and sublethal toxicity occurred at μg/L levels, suggesting the potential risk of these compounds to the environment. Although TCS and TCC have been recently banned from consumer antiseptic wash products by FDA

(Food and Drug Administration), this rule only applies to “consumer wash products” (FDA, 2016). That is, they are continually used in other groups of products such as consumer hand sanitizers or wipes, or antibacterial products used in health care settings. Together with their environmental persistence and bioaccumulation tendency, the potential environmental risks of these compounds should not be neglected. To the best of our knowledge, this is the first study that has systemically examined the toxicity effects of TCS and TCC in a model organism the nematode *C. elegans*. Our findings also suggest that future studies are needed to further understand the underlying mechanisms of the observed toxicity induced by these antimicrobials in this model organism, especially their potential endocrine disruption effects. Future studies should also use lower concentrations to look at effects at molecular/genetic levels and transgenerational effects. Finally, more toxicological studies on TCC is warranted given its greater toxicity potency than TCS yet very limited literature available.

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CHAPTER 4:

# The role of IGF in TCS- and TCC-induced toxicity in the model *C. elegans*


## Abstract

Triclosan (TCS) and triclocarban (TCC) are known endocrine disruptors commonly used in personal care products, plastics, textiles, and medical devices. TCS and TCC are ubiquitously found in the environment and have been detected in tissues and fluids of many organisms. Exposure to TCS and TCC is associated with reproductive and developmental toxicity, increased obesity risk, infertility, and development and proliferation of cancer cells. In our previous study, TCS and TCC caused extended hatching time, decreased reproduction and longevity, and the translocation of *daf-16*, a transcription factor, influenced by IGF signaling in *C. elegans*. However, little is known about the effects of TCS and TCC on IGF signaling or if the IGF signaling pathway plays a role in TCS and TCC toxicity. Here we examined the potential role of IGF signaling in TCS- and TCC-induced toxicity in *C. elegans* via genetic analysis with a series of worm mutants and gene expression in wildtype worms. Our study found that TCS and TCC significantly increased the expression of genes involved in IGF signaling and vitellogenesis. The genetic analysis of IGF-mediated toxicity using mutant strains found that the loss-of-function of *daf-18* was associated with increased toxicity, while the loss or reduction of activity of IGF genes, especially *age-1, pdk-1,* and *akt-1* were associated with decreased toxicity of TCS and TCC. Increased expression of IGF signaling genes was associated with increased acute toxicity, decreased reproduction and lifespan, and increased frequency of an age-related vulval integrity defect (Avid phenotype) in the worm. These findings suggest that IGF plays a role in TCS and TCC-induced toxicity. However, only TCC increased the expression of *daf-2*, the IGF receptor. It is not clear how TCS increases the activity of IGF signaling genes downstream of *daf-2*. Additional research is needed to determine how TCS-induced toxicity affects the

functioning of the IGF pathway by bypassing the IGF receptor, *daf-2*, as well as the role of IGF and vitellogenesis in the development of avid phenotypes.

**Introduction**

Since their introduction in the mid-20$^{th}$ century, triclosan (TCS) and triclocarban (TCC) are commonly used antimicrobials found in numerous personal care, consumer products, and medical devices (i.e., cosmetics, food packaging, plastics, children's toys, clothing, building materials, textiles, and medical devices) (Halden, 2014). TCS and TCC enter the environment through wastewater containing personal care products (i.e., soaps, toothpaste, hair care products, and cosmetics) that are washed down our drains every day (Pycke et al., 2014). The frequent use and ubiquitous detection of TCS and TCC have raised concerns regarding the toxicological effects these chemicals have on human health and the environment (Halden, 2016). These growing concerns have led to numerous *in vivo*, *in vitro*, and epidemiological studies on TCS and TCC, such as their degradation products, bioaccumulation in aquatic plants and animals, partitioning into blood and breast milk, contribution to antibiotic resistance, and increased allergen sensitivity (Halden et al., 2017).

Many studies have shown that TCS and TCC have the ability to bioaccumulate and disrupt endocrine function in a variety of species, including amphibians, fish, and mammals (Dann and Hontela, 2011; Halden et al., 2017). Exposure to TCS and TCC can occur through the absorption, consumption, inhalation and the ingestion of contaminated food, water, particulate matter, soil, sediment, and municipal sludge (Pyke et al., 2014; Olaniyan et al., 2016). Annually, approximately 57 000 ± 233 000 and 140 000 ± 211 000 kg/yr of TCS and TCC, respectively, are applied on U.S. land and crops via the use of sewage sludge, which leads to the absorption of these compounds into crops and their entrance into our waterways via run-off (Halden, 2014; Healy et al., 2017). In freshwater sediment, concentrations of TCS and TCC can reach up to 53,000 μg/kg and 24,000 μg/kg, respectively (Miller et al., 2008; Chalew & Halden, 2009). While TCS and TCC are lipophilic compounds that are likely to settle out of water (Log

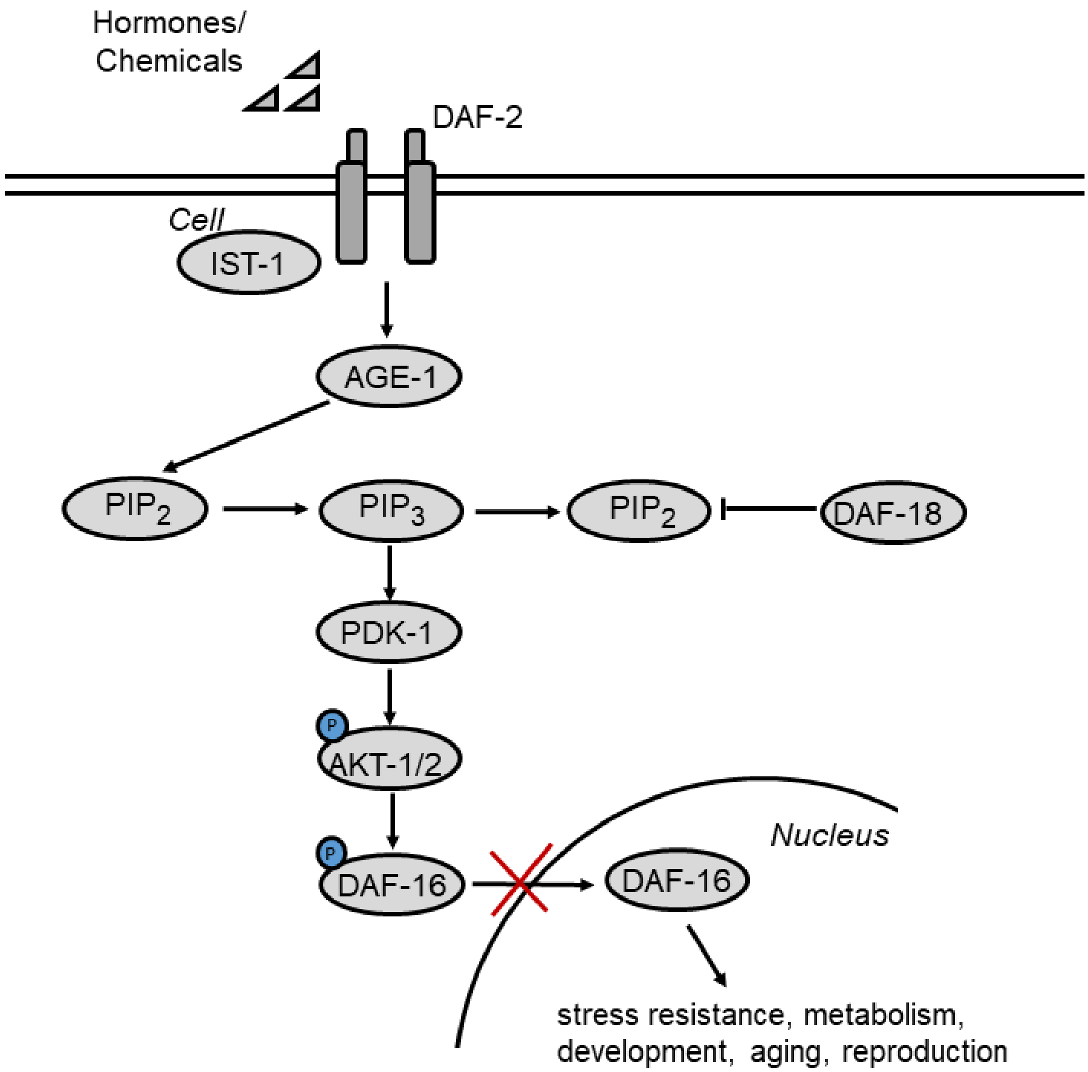
Kow of 4.8 and 4.9, respectively), both TCS and TCC have been detected in freshwater samples across the globe (Coogan & La Point, 2008).

Because of the widespread detection of TCS and TCC, many toxicological studies have been conducted in algae, crustaceans, and fish at environmentally relevant concentrations (Hinther et al., 2011; Tamura et al., 2013; Xu et al., 2015; Halden et al., 2017). These studies have reported that TCS and TCC exhibit acute and sublethal toxicity in aquatic macrobiota at concentrations ranging from 1.4 µg/L to 3,000 µg/L and 0.2 to 440 µg/L, respectively (Chalew & Halden, 2009; von der Ohe et al., 2012; Dan & Hontela, 2011; Rochester et al., 2017). The most widely reported classification of the sublethal effect of TCS and TCC is endocrine disruption (Alfhili and Lee, 2019; Thambirajah et al., 2019). Endocrine disruption is defined as the interference of the endocrine system of an organism by a chemical that leads to a variety of possible adverse effects on development, interference of reproduction, changes in muscle mass, and increased cancer risk that can be mediated through several pathways (i.e., sex hormones, insulin and insulin-like signaling, growth hormones, stress hormones, etc) (Vandenberg et al., 2012; US EPA, 2017). TCS and TCC have been reported to exhibit endocrine disrupting effects in aquatic organisms, rodents, and humans (Dan & Hontela, 2011; Rochester et al., 2017). In aquatic and mammalian organisms, TCS is reported to interfere with hormone activity and metabolism, behavior, development, and reproduction at environmentally relevant concentrations (Chalew & Halden, 2009; Fair et al., 2009; Fritsch et al., 2013, Geiß et al., 2016; Guidice & Young, 2010; Coogan & La Point, 2008; Dan & Hontela, 2011; Rochester et al., 2017).

More recently, the potential endocrine disrupting effects of TCS and TCC have been studied in invertebrates, such as insects, nematodes, and rotifers. A study from 2017 found the potential endocrine disrupting effects of TCS in the invertebrate *Chironomus riparius*. Martinez de Paz and colleagues found that a 24 hour exposure of TCS ranging from 10 to 1,000 µg/L altered the endocrine-related ecdysteroid activity in insect larvae (Martinez de Paz et al., 2017). Another study using *Daphnia* *magna* reported that TCS was reproductively toxic (Orvos et al., 2002). In the monogonont rotifer (Brachionus koreanus) TCS and TCC was acutely toxic (393.1 and 388.1 µg/L, respectively), decreased growth rate and reproduction, and altered the transcriptional regulation of genes controlling oxidative stress (Han et al., 2016). Moreover, studies of TCS and TCC in the nematode *Caenorhabditis elegans* (*C. elegans*) have found that TCS and TCC exposure was associated with developmental and reproductive toxicity, decreased longevity, lipid accumulation, and altered activity of genes involved in the worm's stress response (Lenz et al., 2017; Garcia-Espineira et al., 2018). These findings in *C. elegans* shed light on the potential involvement of the highly conserved insulin-like growth factor (IGF) signaling pathway in TCS- and TCC-induced toxicity. The involvement of IGF in TCS- and TCC-induced toxicity would provide an explanation for the observed lipid accumulation, decreased longevity, and increased *daf-16* activation reported by previous studies in *C. elegans*, as well as similar findings in other organisms (Lenz et al., 2017; Garcia-Espineira et al., 2018).

IGF signaling is a highly conserved endocrine signaling pathway found in organisms from yeast to humans that is well studied in *C. elegans* (Ogawa et al., 2008; Fontana et al., 2010). In the worm, IGF signaling is the "central determinant of endocrine control" of several processes, such as aging, reproduction, stress response, and development (Baumeister et al., 2006). Studies in *C. elegans*, a free-living nematode found in soil and freshwater environments (Hoss & Weltie, 2007), have revealed the mechanistics of IGF signaling (Hunt, 2017). IGF signaling is initiated by the activation of *daf-2* (IGF receptor-1) via ligand (i.e., insulin and insulin-like peptides) engagement with *daf-2* (Fig. 4.1). The activation of *daf-2* triggers a signaling cascade involving activation of *age-1* (phosphoinositide 3-kinase) allowing for the conversion of phosphoinositide-3,4-P2 (*pip2*) to phosphoinositide-3,4,5-P3 (*pip3*). The increase in *pip3* activates *pdk-1* (phosphoinositide-dependent protein kinase-1), leading to the phosphorylation of *akt-1/2* (protein kinase B). The phosphorylation/activation of *akt-1/2* then results in the phosphorylation of *daf-16* (Forkhead box class O), which inhibits its relocation into the nucleus

Figure 4.1. Insulin-like growth factor signaling in *C. elegans*.




The binding of insulin-like peptides activates *daf-2* which in turn activates *age-1* (phosphoinositide 3-kinase). *Age-1* catalyzes the generation of *PIP3* (phosphatidylinositol-3,4,5-trisphosphate) and activates *pdk-1* (phosphoinositide-dependent protein kinase-1) and *akt-1/2* (protein kinase B). The activations of this signaling cascade promotes the phosphorylation of *daf-16* (indicated by circles with a "P") and inhibits its relocation into the nucleus. *daf-18* (PTEN; phosphatase and tensin homolog) reduces *age-1* activity by converting *PIP3* to *PIP2* (phosphatidylinositol 4,5-bisphosphate) (Murphy & Hu, 2005).

(Murphy & Hu, 2005). The phosphorylation of *daf-16* prevents its activation, which is necessary for responding to the presence of a stressor, such as TCS and TCC.

The IGF receptor is a homodimeric, disulphife-linked receptor tyrosine kinase consisting of four binding sites (1, 1′, 2 and 2′) (Surinya et al., 2007; Xu et al., 2018). Interactions between the IGF receptor and ligand occur in a bivalent manner and cross-link two α-subunits, engaging site 1 and then forming a cross-link to site 2’ (Surinya et al., 2007; Xu et al., 2018). IGF-1 and IGF-2, IGF receptor ligands, have one flat surface with aromatic and aliphatic residues (De Meyts et al., 2013; Andersen et al., 2017). Due to the receptor-ligand interactions of IGF, it is possible that other aromatic compounds can bind to the IGF recptor and mimic IGF ligands, such as IGF-1 and IGF-2 (Allard and Duan, 2018). Two studies have shown that the aromatic compounds, benzo-α-pyrene (BαP) and Evans blue (EB), have the ability to interfere with normal IGF singalling (Beery et al., 2001; Fadiel et al., 2013). BαP reduced the expression of IGF-1, an IGF ligand, and weakened the substrate–ligand binding interaction with the IGF receptor (Fadiel et al., 2013). Evans blue (EB), a polysulfonated aromatic compound, activated the IGF receptor and mimicked the effect of IGF by phosphorylating the IGF receptor (*daf-2*). The phosphorylation of the IGF receptor (*daf-2*) resulted in the activation of phosphatidylinositol 3-kinase (*age-1*), phosphoinositide-dependent protein kinase-1 (*pdk-1*), and protein kinase B (*akt-1/2*) cascade of IGF signaling (Fig. 4.1) (Beery et al., 2001).

TCS and TCC have been shown to cause lipid accumulation, decrease longevity and reproduction, and increase *daf-16* activation in *C. elegans* (Lenz et al., 2017; Garcia-Espineira et al., 2018). Studies in *C. elegans* have shown that *daf-16* is involved in IGF signaling (Murphy & Hu, 2005). In addition, the modulation of IGF is essential for the regulation of development, aging, lipid synthesis, and stress response (Baumeister et al., 2006). Both TCS and TCC are well-known endocrine disruptors that impact a variety of hormones (i.e., estrogen, testosterone, and thyroid hormones); however there is very little information on the effects of TCS and TCC on IGF signaling (Halden et al., 2017). We hypothesized that these antimicrobials may elicit toxicity in the worm through impacting the IGF pathway. Given the lack of information regarding if the IGF signaling pathway is involved in TCS- and TCC-induced toxicity in other animal models and humans, it is reasonable to start examining such question using a simple yet biologically and ecologically relevant model *C elegans*. Here we assessed the role of IGF signaling pathway in TCS- and TCC-induced toxicity using the model organism the nematode *C. elegans*. We examined the potential involvement of IGF signaling in TCS- and TCC-induced toxicity using genetic analysis with a series of worm mutants and gene expression. Findings from this study will provide a foundation for the examination of the role of IGF in toxic exposures. This study will address the current knowledge gaps and bring to light the potential endocrine disrupting effect of these widely used antimicrobials.

## Methods and Materials

### Chemicals

TCS (≥97% purity) and TCC (≥98.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (1,000 mg/L dissolved in dimethyl sulfoxide) were used to create all the exposure concentrations with K-medium (51 mM NaCl, 32 mM KCl, pH 6.8) as the diluent. TCS exposure concentrations were 0.01 to 10 mg/L TCC exposure concentrations were 0.001 to 10 mg/L. All sub-lethal concentrations were the same as our previous study (Lenz et al., 2017). The final DMSO concentration was <1% for acute lethality and < 0.5% for sub-lethal exposures.

### ***C. elegans*** **strains**

All C. elegans strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). The strains used for the study of lethality, reproduction, and lifespan are N2 Bristol (wildtype); CB1375 [*daf-18(e1375)*]; CY399 [*sqt-1(sc13) age-1(mg109); pdk-1(mg261)*]; CY400 [*sqt-1(sc13) age-1(mg109) II; akt-1(mg247)*]; DR26 [*daf-16(m26)*]; DR1309 [*daf-16(m26); daf-2(e1370)*];

DR1408 [*daf-16(m26); age-1(m333)*]; DR1572 [*daf-2(e1368)*]; GR1310 [*akt-1(mg144)*]; GR1318 [*pdk-1(mg142)*]; JT9609 [*pdk-1(sa680)*]; RB2621 [*ist-1(ok2706)*]; RB712 [*daf-18(ok480)*]; RB759 [*akt-1(ok525)*]; TJ1052 [*age-1(hx546)*]; VC204 [*akt-2(ok393)*]. All nematodes were maintained on nematode growth medium agar plates seeded with *E. coli* OP50. The *E. coli* OP50 was maintained at 20°C (Stiernagle, 2006). Age-synchronized populations were obtained for acute lethality and sub-lethal assays through the collection of eggs from gravid adult *C. elegans*. The cleaning of eggs was done by bleaching with 1% NaClO and 0.013M NaOH solution and followed by a K-medium wash (Donkin & Williams, 1995).

**Lethality**

Lethality assays were previously described by Lenz and colleagues (2017). Young adult, age-synchronized worms (aged 3 days) were placed in 24-well plates containing 1 mL of exposure solutions made with K-medium. Each exposure consisted of 3 wells containing 10 worms each with an exposure time lasting 24 hours. The exposures consisted of K-medium (negative control), TCS (0.01 to 10 mg/L), or TCC (0.001 mg/L to 10 mg/L) and excluded any feeding agent. After 24 hours, the mortality was scored using a dissecting microscope. The LC50s were calculated using logistic regression of exposure and percent mortality using SigmaPlot (Systat Software, Inc., San Jose, CA).

**Reproduction**

Age-synchronized worms, aged 2 days (L4-staged), were placed on OP50 seeded NGM plates amended with 150 µL of K-medium (negative control), TCS (0.1 and 1 mg/L), or TCC (0.01 mg/L and 0.1 mg/L). NGM exposure plate preparation for exposures was previously described by Lenz and colleagues (2017). Each plate contained one L4-staged worm that was stored at 20 °C for the duration of the exposure. Every other day the parent worm was transferred to a fresh plate (a total of 2-3 transfers over approximately 6 days) and the number of progeny (eggs and larval worms) was recorded. The average number of progeny was calculated by adding the number of progeny from all plates per worm (a total of 3-4 plates per worm) for all exposures.

**Lifespan and vulval integrity**

Lifespan experiments followed the standard protocol, exposing L4-staged worms on NGM plates seeded with OP50 and 0.2 mM 5-fluoro 2-deoxyuridine (FUdR) to inhibit progeny production (Pluskota et al., 2009). Similar to reproduction, plates seeded with OP50 and FUdR were amended with 150 µL of K-medium (negative control), TCS (0.1 and 1 mg/L), or TCC (0.01 mg/L and 0.1 mg/L). Two plates containing 50 worms each were used for each exposure (100 total worms per exposure per replicate). Every other day, dead worms, defined as the failed response to an external stimulus applied to the anterior and posterior ends with a platinum picker, were removed and counted per exposure plate and the total number of dead worms per plate was recorded. At the time of death, the presence of an age-related vulval integrity defect (Avid phenotype), the loss of integrity of the vulva occurring in post-mitotic hermaphrodites (Leiser et al., 2016), were examined upon the death of nematodes (Lenz et al., 2018). Any burrowed worms were excluded from totals.

**RNA Isolation**

Age-synchronized L4 worms were washed with K-medium and exposed for 24 hours on NGM plates amended with 300 µL of K-medium (negative control), TCS (0.1 and 1 mg/L), or TCC (0.01 mg/L and 0.1 mg/L). All exposures were stored at 20°C for 24 hours. Exposed worms were washed with M9 media ($KH_2PO_4$, $Na_2HPO_4$, NaCl, 1 M $MgSO_4$, and nuclease-free $H_2O$) 2-3 times to remove any residual OP50 before 800 µL of TRI reagent (Sigma Aldrich, USA) was added per 0.1 g of worms for RNA isolation. RNA isolation was conducting using the methods described by He (2011). RNA was isolated from worms using freeze-thaw methods consisting of 30 sec of vortexing, freezing in dry ice and methanol, and thaw in a 37°C water bath for a total of 6 times. Following the freeze-thaw of sample, 2 mL of chloroform was added to each sample and mixed for 15 sec. Samples containing chloroform were left at room temperature for 3 minutes to allow phase separation followed by centrifugation for 15 min at 12,000 x g at 4°C. In a fresh tube, the top aqueous phase containing RNA was mixed with 400-500 µl of isopropanol and centrifuged at 12,000 x g at 4°C for 10 min. The remaining RNA pellet was washed with ice cold 75% ethanol and centrifuged a final time at 4,000 rpm at 4°C for 5 min. All ethanol was removed and evaporated before adding 25 µl of nuclease-free $H_2O$ to resuspend the RNA prior to DNase treatment (He, 2011). DNase treatment was conducted according to DNA-free DNA Removal Kit (Invitrogen, USA) using 1 µl of rDNase I with up to 10 µg of RNA in a 50 µl reaction. All RNA was stored at -80°C.

**cDNA synthesis and qPCR**

Complimentary DNA (cDNA) was synthesized using 1 µg of RNA (DNase treated) in a 20 µl reaction according to qScript cDNA synthesis kit (Quanta Biosciences, USA). Our qPCR methods were modified from those reported by Kumar and colleagues (2015). The qPCR reactions were performed as 20 µl reactions containing 1 µl of cDNA (10 ng) using PowerUp SYBR Green PCR Master Mix (Applied Biosystems, USA) and 10 µM of each primer organized in a 96-well format. The thermo-cycling conditions performed by the Roche LightCycler 480 (Roche, USA) included a one cycle of denaturation at 95°C for 2 min, followed by 40 cycles of denaturing at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Actin-1 (*act-1*), housekeeping gene, was used to normalize Ct values of the 21 genes of interest (Table 4.1). The 21 genes were selected based on their involvement in insulin-like growth factor (IGF) signaling, lifespan, development, and bacterial response. Four main categories of genes were used for this study: IGF signaling (e.g., *daf-2, ist-1, age-1, daf-18, pdk-1, akt-1, akt-2,* and *daf-16*), development and longevity (e.g., *let-363, daf-15, rsks-1, sgk-1,* and *skn-1*), development (e.g., *vit-2, vit-5, vit-6, lin-42,* and *daf-12*), and bacterial response (e.g., *clec-60, lys-7,* and *pgp-5*). The relative gene expression was determined using the ΔΔCt method (Kumar et al., 2015).

Table 4.1. List of target genes for gene expression.

| Gene code | Transcript ID | Protein product |
|---|---|---|
| Reference gene | | |
| *act-1* | T04C12.6 | Actin-1 |
| Insulin-like Growth Factor Signalling | | |
| *daf-2* | Y55D5A.5 | Insulin-like receptor 1 |
| *ist-1* | C54D1.3 | Insluin receptor substrate homolog |
| *age-1* | B0334.8 | Phosphatidylinositol 3-kinase |
| *daf-18* | T07A9.6 | Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphtase |
| *pdk-1* | H42K12.1 | 3-phosphoinositide-dependent protein kinase 1 |
| *akt-1* | C12D8.10 | Serine/threonine-protein kinase |
| *akt-2* | F28H6.1 | Serine/threonine-protein kinase |
| *daf-16* | R13H8.1 | Forkhead box protein O |
| Development and Longevity | | |
| *let-363* | B0261.2 | Target of rapamycin homolog |
| *daf-15* | C10C5.6 | Uncharacterized protein on chromosome IV |
| *rsks-1* | Y47D3A.16 | Ribosomal protein S6 kinase beta |
| *sgk-1* | W10G6.2 | Serine/threonine-protein kinase |
| *skn-1* | T19E7.2 | Protein skinhead-1 |
| Development | | |
| *vit-2* | C42D8.2 | Vitellogenin-2 |
| *vit-5* | C04F6.1 | Vitellogenin-5 |
| *vit-6* | K07H8.6 | Vitellogenin-6 |
| *lin-42* | F47F6.1 | Period protein homolog |
| *daf-12* | F11A1.3 | Nuclear hormone receptor |
| Antimicrobial Response | | |
| *clec-60* | ZK666.6 | C-type LECtin |
| *lys-7* | C02A12.4 | Lysozyme-like protein 7 |
| *pgp-5* | C05A9.1 | p-GlycoProtein 5 |

All transcripts tested were broken into four groups: insulin-like growth factor signaling, development and longevity, development, and bacterial response. Gene code indicates the name of respective genes found in *C. elegans*. The protein products indicate the specific protein name commonly used with vertebrates.

**Data analysis**

All assays were repeated three times. One-way analysis of variance was performed for the comparison of differences between individual TCS and TCC exposures compared to controls. Lifespan was analyzed using Kaplan-Meier survival analysis, estimation of the mean lifespan, and log-rank test, comparison of the survival function between individual exposures and between strains. Data analysis was conducted using IBM SPSS Statistics (Armonk, NY).

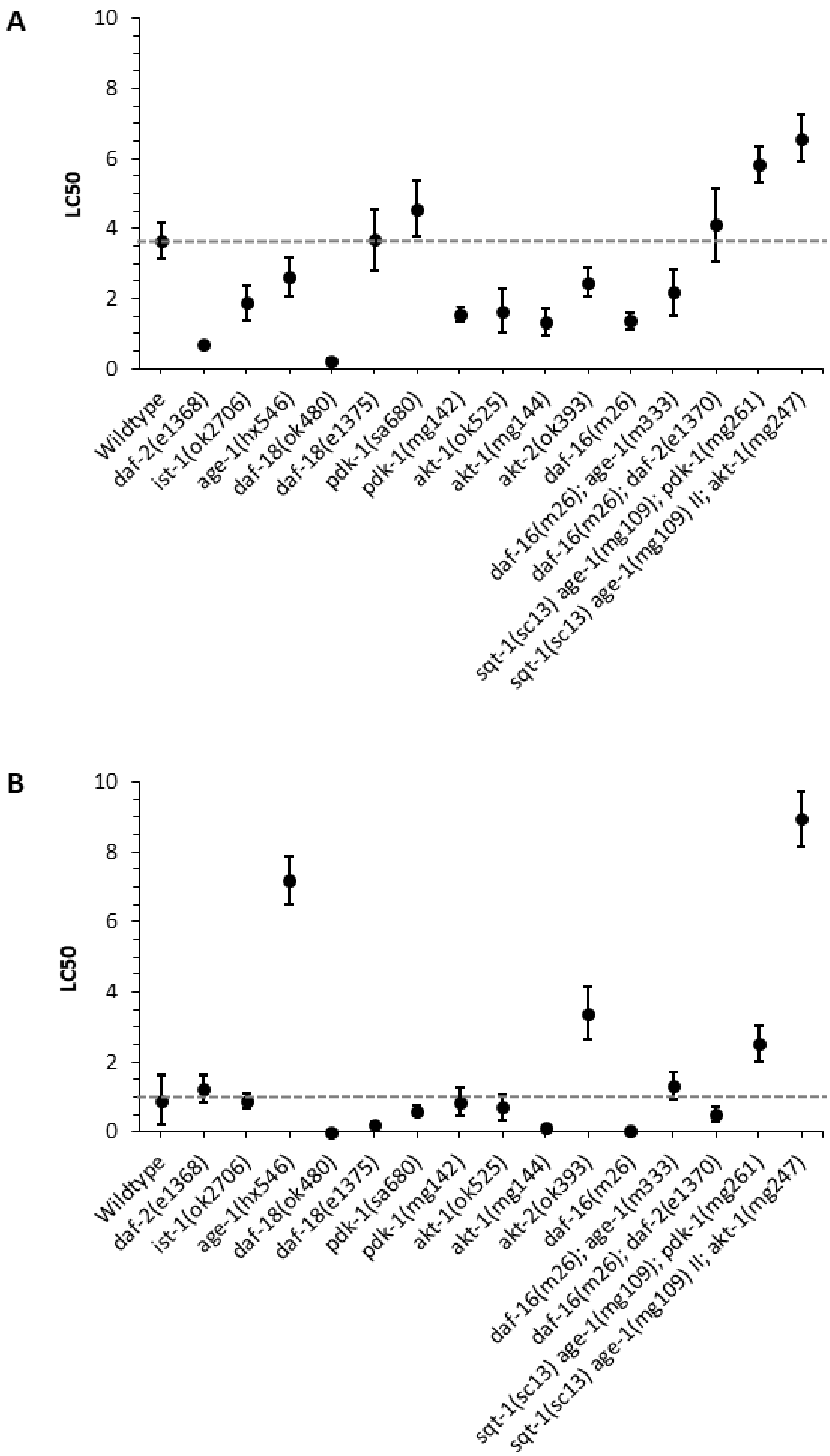
## Results

***Impact of TCS and TCC on lethality***

In *C. elegans*, IGF signaling plays a vital role in development, reproduction, longevity, and stress response. We have previously observed that TCS and TCC had a negative impact on the worm's development, reproduction and longevity, which leads us to ask that if these antimicrobials elicit such toxicity through impacting IGF signaling pathway (Lenz et al., 2017). IGF signaling is initiated by the activation of *daf-2* (IGF receptor-1) via ligand engagement with *daf-2* (Fig. 4.1). The activation of *daf-2* triggers a signaling cascade involving *age-1* (phosphoinositide 3-kinase), *pdk-1* (phosphoinositide-dependent protein kinase-1), phosphorylation of *akt-1/2* (protein kinase B), which result in the phosphorylation of *daf-16* (Forkhead box class O) (Murphy & Hu, 2005). To examine the effects of TCS and TCC on IGF signaling, we conducted a genetic analysis using mutant strains to determine their role of IGF in the observed TCS- and TCC-induced toxicity.

First, we examined the acute lethal toxicity in wildtype *C. elegans.* In wildtype worms, TCS and TCC exposure induced acute lethal toxicity with 24 hour LC50s of 3.7 (95% CI: 3.2, 4.3) and 0.9 (95% CI: 0.5, 1.5) (Lenz et al., 2017). Next, we examined the acute lethal toxicity using *C. elegans* with mutants is genes involved in IGF signaling (e.g., *daf-2*, *ist-1*, *age-1*, *daf-18*, *pdk-1*, *akt-1*, *akt-2*, and *daf-16*). The IGF mutants had functional changes in one or more of the genes involved in IGF signaling to determine the

Figure 4.2. Lethality in IGF mutants.




(A) TCS. (B) TCC. A dashed line indicates the LC50 calculated for wildtype worms. Error bars indicate 95% confidence intervals. The LC50 of TCS and TCC in wildtype worms is 3.7 (95% CI: 3.2, 4.3) and 0.9 (95% CI: 0.5, 1.5), respectively. The lethality of TCS and TCC varied by mutant.

Table 4.2. LC50s of TCS and TCC in IGF mutants.

| | TCS | | TCC | |
|---|---|---|---|---|
| **Gene (allele)** | **LC50 (95% Confidence Interval, mg/L)** | **Difference (mg/L)** | **LC50 (95% Confidence Interval, mg/L)** | **Difference (mg/L)** |
| wildtype | 3.7 (3.2, 4.3) | | 0.9 (0.5, 1.5) | |
| *daf-2(e1368)* | 0.7 (0.6, 0.8) | -3.0 | 1.2 (0.9, 1.6) | 0.3 |
| *ist-1(ok2706)* | 1.9 (1.4, 2.5) | -1.8 | 0.9 (0.7, 1.2) | 0.0 |
| *age-1(hx546)* | 2.6 (2.1, 3.3) | -1.1 | 7.2 (6.5, 8.0) | 6.3 |
| *daf-18(ok480)* | 0.2 (0.1, 0.4) | -3.5 | 0.008 (0.005, 0.01) | -0.9 |
| *daf-18(e1375)* | 3.7 (2.8, 4.6) | 0.0 | 0.2 (0.1, 0.3) | -0.7 |
| *pdk-1(sa680)* | 4.6 (3.8, 5.4) | 0.9 | 0.6 (0.5, 0.8) | -0.3 |
| *pdk-1(mg142)* | 1.5 (1.3, 1.8) | -2.2 | 0.9 (0.4, 1.6) | 0.0 |
| *akt-1(ok525)* | 1.7 (1.0, 2.5) | -2.0 | 0.7 (0.3, 1.4) | -0.2 |
| *akt-1(mg144)* | 1.3 (0.9, 1.9) | -2.4 | 0.13 (0.11, 0.15) | -0.8 |
| *akt-2(ok393)* | 2.5 (2.1, 2.9) | -1.2 | 3.4 (2.7, 4.3) | 2.5 |
| *daf-16(m26)* | 1.4 (1.1, 1.6) | -2.3 | 0.043 (0.042, 0.045) | -0.9 |
| *daf-16(m26); age-1(m333)* | 2.2 (1.5, 3.1) | -1.5 | 1.3 (0.9, 1.8) | 0.4 |
| *daf-16(m26); daf-2(e1370)* | 4.1 (3.1, 5.2) | 0.4 | 0.5 (0.3, 0.8) | -0.4 |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | 5.8 (5.3, 6.3) | 2.1 | 2.5 (2.0, 3.1) | 1.6 |
| *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* | 6.6 (5.9, 7.2) | 2.9 | 8.9 (7.8, 9.6) | 8.0 |

The acute lethality of TCS and TCC was found to vary by mutant and exposure to TCS and TCC. The LC50 of TCS and TCC in wildtype worms is 3.7 (95% CI: 3.2, 4.3) and 0.9 (95% CI: 0.5, 1.5), respectively. Both TCS and TCC were the most toxic in daf-18(ok480) worms with LC50 of 0.2 (0.1, 0.4) and 0.008 (0.005, 0.01), respectively. TCS was found to be less toxic in *pdk-1(sa680), daf-16(m26); daf-2(e1370), sqt-1(sc13) age-1(mg109); pdk-1(mg261),* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms. TCC was found to be less toxic in *daf-2(e1368), age-1(hx546), akt-2(ok393), sqt-1(sc13) age-1(mg109); pdk-1(mg261),* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms.

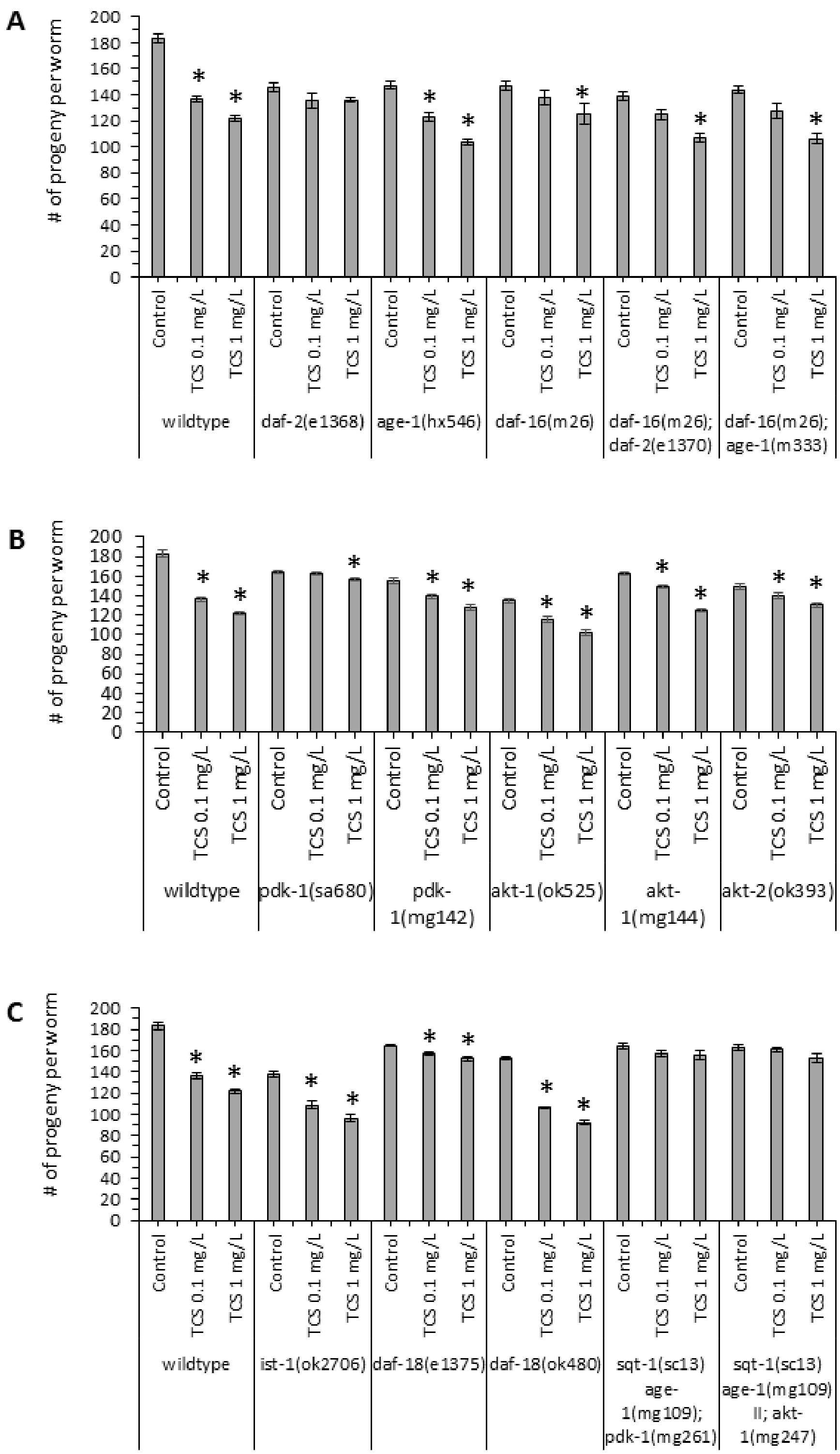
role each gene played in the observed TCS- and TCC-induced toxicity. Single gene mutants allow for observations of the role of specific gene in TCS- and TCC-induced toxicity, while mutants allows for observations of genes intercactions (Fig. 4.2 and Table 4.2).

In the IGF mutants, increased acute lethal toxicity of both TCS and TCC were observed in *daf-18(ok480), akt-1(ok525), akt-1(mg144),* and *daf-16(m26)* worms. The LC50 of TCS in *daf-18(ok480), akt-1(ok525), akt-1(mg144),* and *daf-16(m26)* worms ranged from 0.2 to 1.7 mg/L, while the LC50 of TCC ranged from 0.008 to 0.7 mg/L in the worms (Table 4.2). Other mutant strains exhibited increased acute toxicity when exposed to TCS or TCC. TCS also increased the acute toxicity (LC50s ranging from 0.7 to 2.6 mg/L) of *daf-2(e1368), ist-1(ok2706), age-1(hx546), pdk-1(mg142), akt-2(ok393),* and *daf-16(m26); age-1(m333)* worms, while TCC was more acutely toxic (LC50s ranging from 0.2 to 0.7 mg/L) in *daf-18(e1375), pdk-1(sa680), akt-1(ok525),* and *daf-16(m26); daf-2(e1370)* worms comparable to wildtype worms (Fig. 4.2 and Table 4.2).

***Impact of TCS and TCC on reproduction***

Both TCS and TCC were found to show reproductive toxicity *in C. elegans*. In wildtype worms, a TCS (1mg/L) decreased the total number of progeny up to 33.4% (Fig. 4.3 and Table 4.3); while TCC (0.1 mg/L) decreased the total number of progeny by up to 38.1% (Fig. 4.4 and Table 4.3). This is consistent with our previous study that TCC exposure was more reproductively toxic than TCS in wildtype worms (Lenz et al., 2017). However, reproductive toxicity of TCS and TCC varied in the mutant strains (Table 3). TCC was more reproductively toxic than TCS in *daf-2(e1368), daf-18(ok480), pdk-1(mg142), akt-1(mg144), akt-2(ok393), daf-16(m26),* and *daf-16(m26); daf-2(e1370)* worms. Meanwhile, TCS and TCC had similar trends in reproductive toxicity in *ist-1(ok2706), age-1(hx546), daf-18(e1375), pdk-1(sa680), akt-1(ok525), daf-16(m26); age-1(m333), sqt-1(sc13) age-1(mg109) II; akt-1(mg247),* and *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* worms (Table 4.3).

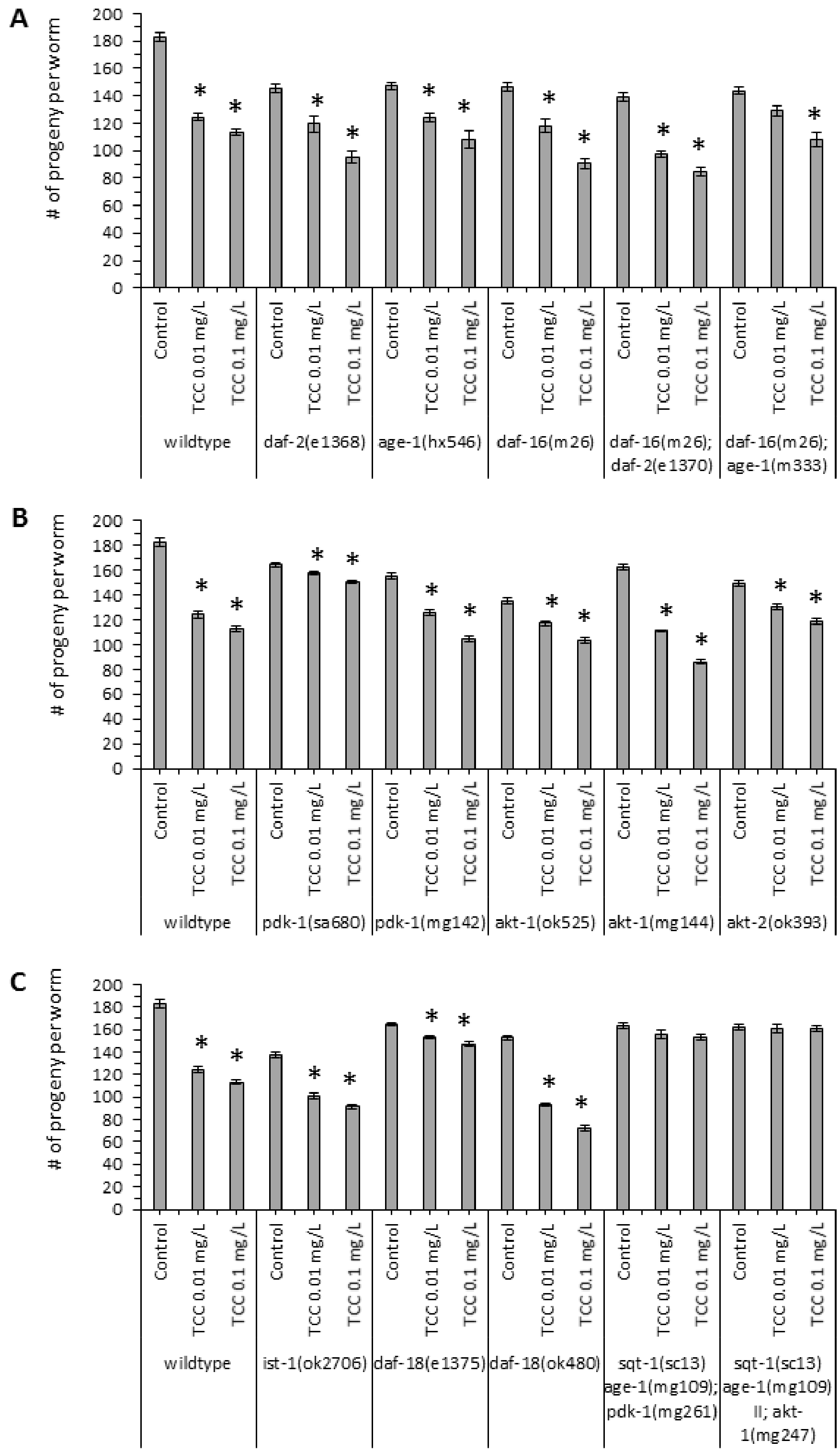
Figure 4.3. Impact TCS on reproduction in IGF mutants.




(A) *daf-2, age-1,* and *daf-16* mutants. (B) *pdk-1* and *akt-1/2* mutants. (C) *ist-1, daf-18, age-1/pdk-1,* and *age-1/akt-1* mutants. Error bars represent standard error. "*" indicates significant difference from controls ($p<0.05$). A 0.1 mg/L and 1 mg/L exposure of TCS decreased the total number of progeny by

25.6% and 33.4%, respectively, compared to controls 183.0 (95% CI: 177.3, 189.8). TCS was less reproductively toxic in *daf-2(e1368), age-1(hx546), daf-18(e1375), pdk-1(sa680), pdk-1(mg142), akt-1(ok525), akt-1(mg144), akt-2(ok393), daf-16(m26), daf-16(m26); age-1(m333), daf-16(m26); daf-2(e1370), sqt-1(sc13) age-1(mg109); pdk-1(mg261),* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms compared to wildtype worms. TCS exhibited the most reproductive toxicity in *daf-18(ok480)* worms.

Figure 4.4. Impact of TCC on reproduction in IGF mutants.




(A) *daf-2, age-1,* and *daf-16* mutants. (B) *pdk-1* and *akt-1/2* mutants. (C) *ist-1, daf-18, age-1/pdk-1,* and *age-1/akt-1* mutants. Error bars represent standard error. "*" indicates significant difference from controls ($p<0.05$). A 0.01 mg/L and 0.1 mg/L exposure of TCC decreased the total number of progeny by

32.0% and 38.1%, respectively, compared to controls 183.0 (95% CI: 177.3, 189.8). TCC was less reproductively toxic in *age-1(hx546), daf-18(e1375), pdk-1(sa680), pdk-1(mg142), akt-1(ok525), akt-2(ok393), daf-16(m26); age-1(m333), sqt-1(sc13) age-1(mg109); pdk-1(mg261),* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms compared to wildtype worms. TCC exhibited the most reproductive toxicity in *daf-18(ok480)* worms.

Table 4.3. Mean number of progeny of IGF mutants.

| | | TCS | | | | TCC | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 0.1 mg/L | | 1 mg/L | | 0.01 mg/L | | 0.1 mg/L | |
| Gene (allele) | Mean # of progeny | Mean # of progeny | % Difference | Mean # of progeny | % Difference | Mean # of progeny | % Difference | Mean # of progeny | % Difference |
| wildtype | 183.0 (177.3, 189.8) | 136.2 (132.3, 140.1)* | -25.6% | 121.8 (118.8, 124.8)* | -33.4% | 124.4 (119.9, 128.9)* | -32.0% | 113.2 (109.7, 116.7)* | -38.1% |
| *daf-2(e1368)* | 153.7 (145.5, 161.8) | 135.7 (122.3, 149.0) | -11.7% | 136.1 (131.2, 141.0) | -11.5% | 119.4 (106.4, 132.5)* | -22.3% | 95.4 (87.0, 103.8)* | -37.9% |
| *ist-1(ok2706)* | 137.6 (132.2, 143.0) | 108.6 (101.8, 115.3)* | -21.1% | 96.1 (88.6, 103.6)* | -30.2% | 100.9 (94.8, 107.0)* | -26.7% | 91.6 (86.9, 96.2)* | -33.4% |
| *age-1(hx546)* | 146.8 (137.5, 156.0) | 122.8 (116.2, 129.5)* | -16.3% | 103.7 (99.2, 108.2)* | -29.4% | 124.3 (117.5, 131.2)* | -15.3% | 108.1 (94.2, 122.0)* | -26.4% |
| *daf-18(ok480)* | 153.0 (149.9, 156.1) | 106.4 (104.4, 108.4)* | -30.5% | 92.1 (86.9, 97.2)* | -39.8% | 92.8 (90.1, 95.4)* | -39.3% | 72.1 (67.3, 76.8)* | -52.9% |
| *daf-18(e1375)* | 164.7 (162.6, 166.8) | 157.2 (154.0, 160.3)* | -4.6% | 152.4 (148.5, 156.2)* | -7.5% | 153.1 (150.8, 155.3)* | -7.0% | 147.1 (144.1, 150.1)* | -10.7% |
| *pdk-1(sa680)* | 165.1 (161.6, 168.7) | 162.4 (159.0, 165.9) | -1.6% | 156.6 (152.4, 160.7)* | -5.1% | 157.6 (154.6, 160.6)* | -4.5% | 150.7 (148.3, 153.1)* | -8.7% |
| *pdk-1(mg142)* | 155.1 (149.7, 160.5) | 139.4 (134.8, 144.1)* | -10.1% | 127.6 (120.2, 135.0) * | -17.7% | 125.7 (121.1, 130.4)* | -19.0% | 105.2 (99.9, 110.4)* | -32.2% |
| *akt-1(ok525)* | 134.9 (129.8, 140.1) | 115.4 (109.5, 121.3)* | -14.5% | 102.1 (95.9, 108.4)* | -24.3% | 117.3 (112.7, 121.9)* | -13.0% | 103.5 (98.9, 108.1)* | -23.3% |
| *akt-1(mg144)* | 163.5 (159.5, 167.4) | 149.1 (146.1, 152.0)* | -8.8% | 124.7 (122.4, 127.0)* | -23.7% | 111.3 (109.2, 113.4)* | -31.9% | 86.0 (82.0, 90.0)* | -47.4% |
| *akt-2(ok393)* | 149.8 (143.7, 155.9) | 139.8 (133.7, 146.0)* | -6.7% | 130.6 (125.6, 135.7)* | -12.8% | 130.7 (126.3, 135.0)* | -12.8% | 119.1 (114.9, 123.2) * | -20.5% |
| *daf-16(m26)* | 152.1 (141.2, 163.1) | 137.7 (125.3, 150.0) | -9.5% | 125.3 (108.6, 141.9)* | -17.6% | 118.3 (107.6, 128.9)* | -22.2% | 90.6 (82.3, 98.9)* | -40.4% |
| *daf-16(m26); age-1(m333)* | 143.3 (135.4, 151.1) | 127.3 (116.1, 138.5) | -11.2% | 106.4 (98.4, 114.3)* | -25.8% | 129.0 (121.7, 136.3) | -10.0% | 108.2 (97.7, 118.7)* | -24.5% |
| *daf-16(m26); daf-2(e1370)* | 137.3 (128.4, 146.3) | 124.9 (113.4, 136.3) | -9.0% | 106.9 (99.9, 114.0)* | -22.1% | 97.4 (92.2, 102.6)* | -29.1% | 84.8 (78.0, 91.6)* | -38.2% |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | 163.2 (157.1, 169.3) | 156.7 (150.4, 163.0) | -4.0% | 155.8 (146.7, 164.9) | -4.5% | 155.7 (147.2, 164.3) | -4.6% | 153.1 (148.3, 158.0) | -6.2% |
| *sqt-1(sc13)) age-1(mg109) II; akt-1(mg247* | 163.2 (157.6, 168.9) | 160.6 (155.0, 166.2) | -1.6% | 152.9 (145.1, 160.7) | -6.3% | 161.0 (153.8, 168.2) | -1.3% | 158.5 (152.0, 165.0) | -2.9% |

"*" indicates significant difference from controls (p<0.05). Both TCS and TCC had significant decreased reproductive toxicity in *daf-18(e1375), pdk-1(sa680), sqt-1(sc13) age-1(mg109); pdk-1(mg261)*, and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* compared to wildtype worms. Both TCS and TCC were the most toxic in *daf-18(ok480)* worms.

The largest decrease in progeny number by both TCS and TCC was observed in *daf-18(ok480)* worms. TCS (1 mg/L) and TCC (0.1 mg/L) decreased the progeny number of *daf-18(ok480)* worms by 40% and 53%, respectively, compared to the 33% and 38% respective decrease in progeny number of wildtype worms (Table 4.3). However, TCS and TCC decreased the total number of progeny in *daf-18(e1375)* worms by <11%. This difference is due to the fact *daf-18(ok480)* has no detectable levels of the DAF-18/PTEN protein that is associated with increased mortaility and avid development (Ogg & Ruvkun, 1998; Fukuyama et al., 2006; Brisbin et al., 2009). In addition, both TCS and TCC had the least observed effect on progeny number in *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms, causing a <7% decrease in progeny number compared to controls (Table 4.3). This suggests that the mutations affecting IGF signaling in these strains may provide protection against the reproductive effects of TCS and TCC.

***Impact of TCS and TCC on lifespan and vulval integrity***

In addition to the acute lethality and reproductive toxicity of TCS and TCC, both compounds have been shown to decrease the lifespan of wildtype *C. elegans*. TCS and TCC decreased the lifespan of *C. elegans* by >15% compared to control worms [17.2 (95% CI: 17.0, 17.3)] (Tables 4.4 and 4.5). In mutant strains, the effects of TCS and TCC varied. However, both TCS and TCC were observed to be less toxic in *ist-1(ok2706), akt-1(ok525), akt-1(mg144), akt-2(ok393), daf-16(m26), daf-16(m26); age-1(m333),* and *daf-16(m26); daf-2(e1370)* worms (Table 4.4 and 4.5). Furthermore, TCS caused a <10% decrease in lifespan of *age-1(hx546), daf-18(ok480)* and *akt-2(ok393*) worms (Table 4.4), while TCC caused a <10% decrease in lifespan in *daf-16(m26), daf-16(m26); age-1(m333), daf-16(m26); daf-2(e1370),* and *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* worms (Table 4.5).

In *ist-1(ok2706), daf-18(ok480), daf-18(e1375),* and *akt-1(mg144)* control worms, an average lifespan of 11.8 to 14.4 days was observed compared to 17.2 (95% CI: 17.0, 17.3) for wildtype controls.

Table 4.4. Impact of TCS on lifespan in IGF mutants.

| | Control | TCS | | | |
|---|---|---|---|---|---|
| | | 0.1 mg/L | | 1 mg/L | |
| Gene (allele) | Mean lifespan (days) | Mean lifespan (days) | Difference (days; %) | Mean lifespan (days) | Difference (days; %) |
| wildtype | 17.2 (17.0, 17.3) | 16.0 (15.8, 16.2)* | -1.2 (-7.0%) | 14.6 (14.4, 14.9)* | -2.6 (-15.1%) |
| *daf-2(e1368)* | 22.2 (21.7, 22.7) | 21.1 (20.5, 21.6)* | -1.1 (-5.0%) | 19.7 (19.1, 20.4)* | -2.5 (-11.3%) |
| *ist-1(ok2706)* | 12.0 (11.8, 12.3) | 11.4 (11.2, 11.6)* | -0.6 (-5.0%) | 11.4 (11.2, 11.6)* | -0.6 (-5.0%) |
| *age-1(hx546)* | 22.3 (21.7, 22.8) | 21.3 (20.9, 21.8) | -1.0 (-4.5%) | 20.9 (20.4, 21.5)* | -1.4 (-6.3%) |
| *daf-18(ok480)* | 14.4 (14.1, 14.7) | 12.9 (12.7, 13.1)* | -1.5 (-10.4%) | 11.9 (11.6, 12.1)* | -2.5 (-17.4%) |
| *daf-18(e1375)* | 13.9 (13.6, 14.2) | 10.9 (10.6, 11.2)* | -3.0 (-21.6%) | 10.6 (10.3, 10.9)* | -3.3 (-23.7%) |
| *pdk-1(sa680)* | 23.6 (23.1, 24.0) | 19.7 (19.2, 20.1)* | -3.9 (-16.5%) | 19.1 (18.6, 19.6)* | -4.5 (-19.1%) |
| *pdk-1(mg142)* | 15.5 (15.2, 15.9) | 14.7 (14.4, 15.0)* | -0.8 (-5.2%) | 13.7 (13.4, 13.9)* | -1.8 (-11.6%) |
| *akt-1(ok525)* | 20.3 (19.7, 20.9) | 18.8 (18.3, 19.4)* | -1.5 (-7.4%) | 17.9 (17.3, 18.4)* | -2.4 (-11.8%) |
| *akt-1(mg144)* | 11.8 (11.6, 12.1) | 10.5 (10.3, 10.7)* | -1.3 (-11.0%) | 10.2 (10.0, 10.7)* | -1.6 (-13.6%) |
| *akt-2(ok393)* | 20.1 (19.5, 20.8) | 18.3 (17.7, 18.9)* | -1.8 (-9.0%) | 18.2 (17.6, 18.9)* | -1.9 (-9.5%) |
| *daf-16(m26)* | 15.9 (15.6, 16.2) | 15.3 (14.9, 15.8) | -0.6 (-3.8%) | 13.9 (13.6, 14.3)* | -2.0 (-12.6%) |
| *daf-16(m26); age-1(m333)* | 15.9 (15.7, 16.1) | 15.6 (15.3, 15.9) | -0.3 (-1.9%) | 14.2 (13.9, 14.4)* | -1.7 (-10.7%) |
| *daf-16(m26); daf-2(e1370)* | 15.9 (15.7, 16.1) | 14.3 (14.0, 14.5)* | -1.6 (-10.1%) | 14.1 (13.8, 14.3)* | -1.8 (-11.3%) |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | 21.6 (21.1, 22.1) | 19.9 (19.3, 20.5)* | -1.7 (-7.9%) | 17.3 (16.6, 17.9)* | -4.3 (-19.9%) |
| *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* | 21.3 (20.8, 21.9) | 19.1 (18.5, 19.6)* | -2.2 (-10.3%) | 18.2 (17.6, 18.7)* | -3.1 (-14.6%) |

"*" indicates significant difference from controls (p<0.05). TCS decreased the lifespan of wildtype C. elegans by up to 15% compared to controls [17.2 (95% CI: 17.0, 17.3)]. TCS decreased the lifespan of wildtype C. elegans by <16% compared to controls [17.2 (95% CI: 17.0, 17.3)]. TCC decreased the average lifespan of *ist-1(ok2706), age-1(hx546),* and *akt-2(ok393)* by <10%, compared to controls.

Table 4.5. Impact of TCC on lifespan in IGF mutants.

| | Control | TCC | | | |
|---|---|---|---|---|---|
| | | 0.01 mg/L | | 0.1 mg/L | |
| Gene (allele) | Mean lifespan (days) | Mean lifespan (days) | Difference (days; %) | Mean lifespan (days) | Difference (days; %) |
| wildtype | 17.2 (17.0, 17.3) | 15.7 (15.5, 15.9)* | -1.5 (-8.7%) | 14.5 (14.3, 14.8)* | -2.7 (-15.7%) |
| *daf-2(e1368)* | 22.2 (21.7, 22.7) | 19.2 (18.8, 19.6)* | -3.0 (-13.5%) | 18.3 (17.9, 18.7)* | -3.9 (-17.6%) |
| *ist-1(ok2706)* | 12.0 (11.8, 12.3) | 11.5 (11.2, 11.7)* | -0.5 (-4.2%) | 10.4 (10.2, 10.6)* | -1.6 (-13.3%) |
| *age-1(hx546)* | 22.3 (21.7, 22.8) | 19.4 (18.9, 19.8)* | -2.9 (-13.0%) | 18.9 (18.5, 19.3)* | -3.4 (-15.2%) |
| *daf-18(ok480)* | 14.4 (14.1, 14.7) | 13.1 (12.9, 13.3)* | -1.3 (-9.0%) | 11.8 (11.7, 12.0)* | -2.6 (-18.11%) |
| *daf-18(e1375)* | 13.9 (13.6, 14.2) | 11.2 (11.0, 11.4)* | -2.7 (-19.4%) | 10.8 (10.6, 10.9)* | -3.1 (-22.3%) |
| *pdk-1(sa680)* | 23.6 (23.1, 24.0) | 20.9 (20.4, 21.3)* | -2.7 (-11.4%) | 18.3 (17.9, 18.8)* | -5.3 (-22.3%) |
| *pdk-1(mg142)* | 15.5 (15.2, 15.9) | 13.7 (13.4, 14.1)* | -1.8 (-11.6%) | 13.3 (13.0, 13.5)* | -2.2 (-14.2%) |
| *akt-1(ok525)* | 20.3 (19.7, 20.9) | 17.3 (16.8, 17.9)* | -3.0 (-14.8%) | 18.0 (17.5, 18.6)* | -2.3 (-11.3%) |
| *akt-1(mg144)* | 11.8 (11.6, 12.1) | 10.3 (10.1, 10.5)* | -1.5 (-12.7%) | 10.2 (10.0, 10.4)* | -1.6 (-13.6%) |
| *akt-2(ok393)* | 20.1 (19.5, 20.8) | 18.8 (18.4, 19.2)* | -1.3 (-6.5%) | 18.1 (17.6, 18.6)* | -2.0 (-10.0%) |
| *daf-16(m26)* | 15.9 (15.6, 16.2) | 15.3 (15, 15.7) | -0.6 (-3.8%) | 14.5 (14.2, 14.8)* | -1.4 (-8.8%) |
| *daf-16(m26); age-1(m333)* | 15.9 (15.7, 16.1) | 14.8 (14.5, 15.1)* | -1.1 (-6.9%) | 13.9 (13.7, 14.2)* | -2.0 (-12.6%) |
| *daf-16(m26); daf-2(e1370)* | 15.9 (15.7, 16.1) | 14.1 (13.9, 14.4)* | -1.8 (-11.3%) | 13.1 (12.6, 13.3)* | -2.8 (-17.6%) |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | 21.6 (21.1, 22.1) | 20.4 (19.9, 21.0)* | -1.2 (-5.6%) | 19.9 (19.3, 20.4)* | -1.7 (-7.9%) |
| *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* | 21.3 (20.8, 21.9) | 20.5 (19.9, 21.0) | -0.8 (-3.8%) | 19.1 (19.4, 19.9)* | -2.2 (-10.3%) |

"*" indicates significant difference from controls ($p<0.05$). TCC decreased the lifespan of wildtype C. elegans by <16% compared to controls [17.2 (95% CI: 17.0, 17.3)]. TCC decreased the average lifespan of *akt-2(ok393), daf-16(m26),* and *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* by ≤10%, compared to controls.

Table 4.6. Impact of TCS and TCC on avid phenotypes in IGF mutants.

| | | TCS | | TCC | |
|---|---|---|---|---|---|
| **Gene (allele)** | **Control** | **0.1 mg/L** | **1 mg/L** | **0.01 mg/L** | **0.1 mg/L** |
| wildtype | 5.6% | 5.3% | 5.3% | 5.4% | 6.5% |
| *daf-2(e1368)* | 4.2% | 4.6% | 6.0%* | 4.4% | 7.0%* |
| *ist-1(ok2706)* | 28.0% | 63.5%* | 65.0%* | 57.5%* | 60.3%* |
| *age-1(hx546)* | 6.4% | 8.0% | 19.0%* | 16.2%* | 18.6%* |
| *daf-18(ok480)* | 22.8% | 40.8%* | 45.0%* | 52.5%* | 61.0%* |
| *daf-18(e1375)* | 36.5% | 44.5%* | 56.0%* | 60.5%* | 68.0%* |
| *pdk-1(sa680)* | 6.7% | 8.7% | 10.0% | 8.7% | 10.3% |
| *pdk-1(mg142)* | 6.5% | 13.8% | 18.3%* | 11.8% | 19.8%* |
| *akt-1(ok525)* | 8.5% | 32.0%* | 44.5%* | 21.0% | 29.5%* |
| *akt-1(mg144)* | 27.0% | 35.0% | 45.0%* | 42.7%* | 56.3%* |
| *akt-2(ok393)* | 6.2% | 10.7% | 17.7%* | 9.8% | 9.0% |
| *daf-16(m26)* | 5.3% | 6.7% | 11.7% | 9.0% | 14.7% |
| *daf-16(m26); age-1(m333)* | 5.0% | 5.7% | 7.3% | 7.3% | 7.7% |
| *daf-16(m26); daf-2(e1370)* | 8.3% | 22.3%* | 33.7%* | 27.7%* | 35.0%* |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | 5.0% | 4.3% | 4.5% | 4.0% | 5.5% |
| *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* | 4.5% | 4.5% | 5.8% | 4.3% | 6.5% |

"*" indicates significant difference from controls (p<0.05). Vulval integrity defects were observed through the presence of "vulval protrusions" that were categorized as one of three Avid types, previously described by Leiser and colleagues (2016). TCS and TCC increased frequency of Avid phenotype in *ist-1(ok2706), age-1(hx546), daf-18(ok480), daf-18(e1375), akt-1(ok525), akt-1(mg144), pdk-1(mg142), daf-16(m26), daf-16(m26); daf-2(e1370)* worms. Vulval integrity defects were the highest (≥45%) at the highest concetration in *ist-1(ok2706), daf-18(ok480), daf-18(e1375), and akt-1(mg144)* worms, which was associated with a significant decrease in mean lifespan (Table 4 and 5).

This decrease in lifespan was accompanied by a >20% frequency in an avid (age-related vulval integrity defect) phenotype, 2-4 days prior to death (Table 4.6). Both TCS and TCC caused an increased frequency of the avid phenotype in *ist-1(ok2706), daf-18(ok480), daf-18(e1375),* and *akt-1(mg144)* worms. TCS exposure (1 mg/L) induced an avid phenotype in 65% of *ist-1(ok2706),* which is the highest observed frequency of this phenotype by TCS. The highest frequency of the avid phenotype in worms exposed to TCC (0.1 mg/L) was 68% in *daf-18(e1375)* worms, followed by 61% in *daf-18(ok480)* and 60.3% in *ist-1(ok2706)* worms (Table 4.6). The high frequency of avid phenotype observed in *daf-18(ok480)* and *daf-18(e1375)* worms was accompanied by a 17.4% to 23.7% decrease in lifespan by TCS and TCC (Tables 4.4 and 4.5). Additionally, TCC decreased the lifespan of *ist-1(ok2706)* worms by 13.3% (Table 4.5), while TCS did not significantly decrease the lifespan of *ist-1(ok2706)* worms (Table 4.4).

***Impact of TCS and TCC on gene expression***

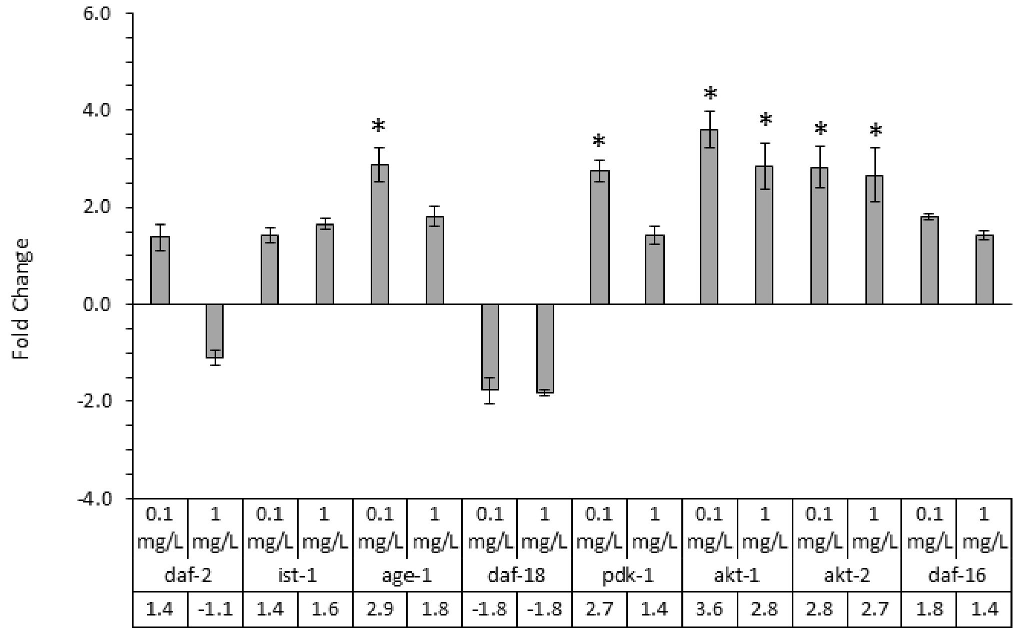
In addition to the use of mutant *C. elegan* strains, we performed qPCR to determine how TCS and TCC exposure impact the expression of specific genes. The selection of the genes were based on the observations from the lethality, reproduction, lifespan and vulval integrity assays. The selected genes were classified based on four main categories based on the genes function: IGF signaling (e.g., *daf-2, ist-1, age-1, daf-18, pdk-1, akt-1, akt-2,* and *daf-16*), development and longevity (e.g., *let-363, daf-15, rsks-1, sgk-1,* and *skn-1*), development (e.g., *vit-2, vit-5, vit-6, lin-42,* and *daf-12*), and bacterial response (e.g., *clec-60, lys-7,* and *pgp-5*) (Table 4.1).

*IGF signaling*

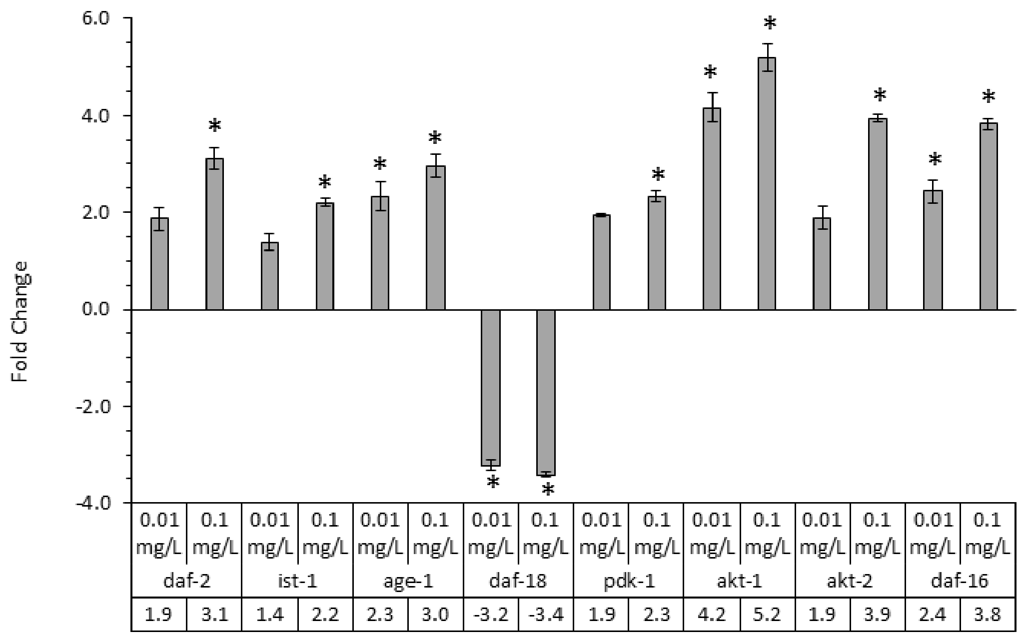
In a previous study, TCS and TCC exposure decreased reproduction and lifespan, while increased the activation of *daf-16*, or the relocation of *daf-16* into the nucleus of cells (Lenz et al., 2017). The activation of *daf-16* is dependent on the expression of IGF signaling genes, such as *daf-2, age-1, pdk-1,* and *akt-1/2*. In this study, we examined the expression of genes involved in IGF signaling and found that

Figure 4.5. Expression of IGF genes exposed to TCS and TCC.

A




B




(A) TCS. (B) TCC. Error bars represent standard error. "*" indicates significant difference from controls ($p<0.05$). Both TCS and TCC exposure lead to an increase in the expression of *age-1*, *pdk-1*, *akt-1*, and *akt-2*. TCC exposure increased the expression of IGF genes to more of an extant than TCS. TCC exposure significantly increased the expression of *daf-2*, *ist-1* and *daf-16*, while significantly decreasing the expression of *daf-18*.

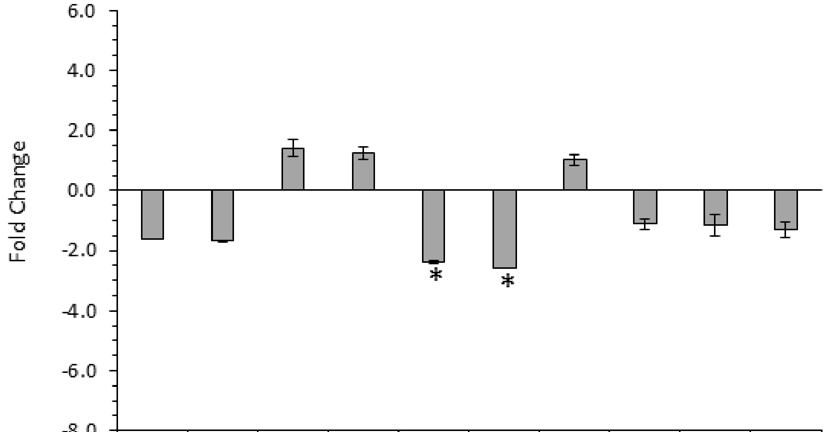
both TCS and TCC exposure lead to an increase in the expression of *age-1*, *pdk-1*, *akt-1*, and *akt-2* (Fig. 4.5). TCC exposure also increased the expression of these four genes (*age-1*, *pdk-1*, *akt-1*, and *akt*-2) by a greater extent than TCS. At 0.1 mg/L, TCC exposure increased *age-1*, *pdk-1*, *akt-1*, and *akt-2* by 3.0, 2.3, 5.2, and 3.9 fold, respectively. In contrast, the lower concentration of TCS had a greater effect on gene expression than the higher concentration. At 0.1 mg/L, TCS increased *age-1*, *pdk-1*, *akt-1*, and *akt-2* expression by 2.9, 2.7, 3.6, and 2.8 fold, respectively. Additionally, both TCS and TCC exposure decreased the expression of *daf-18*, but TCS exposure decreased *daf-18* expression by less of an extent (<2 fold decrease) compared TCC (>3 fold decrease). The deceased expression of *daf-18* with TCC exposure was associated with an increased expression of *daf-2*, which is a consistent relationship of *daf-2* and *daf-18* (Liu et al., 2014). The increased expression of *daf-2*, as well as *ist-1* and *daf-16*, was only observed with exposure to TCC. The expression of *daf-2*, *ist-1*, and *daf-16* were increased by 3.1, 2.2, and 3.8 fold, respectively, when exposed to TCC at 0.1 mg/L (Fig. 4.5). Both TCS and TCC increased the expression of genes downstream of the IGF receptor (*daf-2*); however only TCC increased the expression of *daf-2*.

*Development and longevity*

TCS was found to increase the expression of genes involved in IGF signalling (e.g., *age-1*, *pdk-1*, *akt-1*, and *akt-2*), but TCS did not increase the expression of *daf-2*, the IGF receptor (Fig. 4.5). Due to the increased expression of *pdk-1* and *akt-1/2*, we examined the expression of genes downstream of *pdk-1* and *akt-1/2* that do not influence daf-16 expression, such as *sgk-1* and *skn-1* in our study (Murphy & Hu, 2005). In addition, we considered the possibility that the expression of *akt-1/2* may be influenced by the target of rapamycin (TOR; *let-363*), a member of phosphatidylinositol kinase-related kinase family and raptor (*daf-15*), a binding partner of TOR (Jia et al., 2004). The modulation of *daf-15* and *let-363* expression are associated with decreased lifespan and fat accumulation, which may provide insight in the development of avids in certain mutants (Table 4.6) (Jia et al., 2004). Lastly, *rsks-1* works in parallel

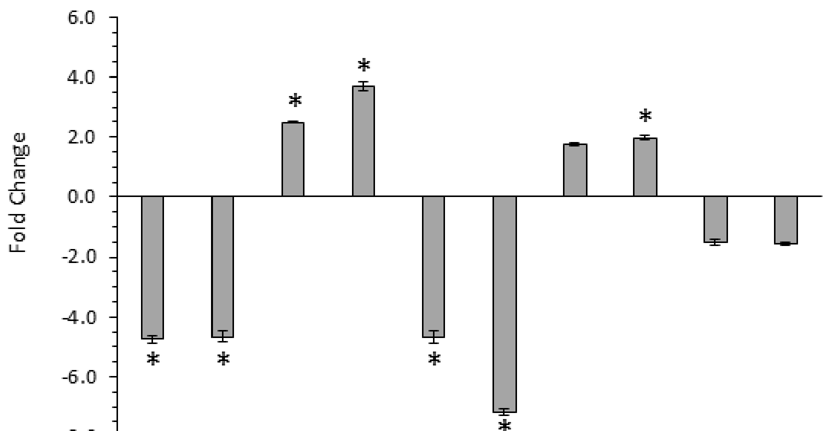
Figure 4.6. Expression of genes controlling development and longevity exposed to TCS and TCC.

**A**




| let-363 | | daf-15 | | rsks-1 | | sgk-1 | | skn-1 | |
|---|---|---|---|---|---|---|---|---|---|
| 0.1 mg/L | 1 mg/L | 0.1 mg/L | 1 mg/L | 0.1 mg/L | 1 mg/L | 0.1 mg/L | 1 mg/L | 0.1 mg/L | 1 mg/L |
| -1.6 | -1.7 | 1.4 | 1.2 | -2.4 | -2.6 | 1.0 | -1.1 | -1.2 | -1.3 |

**B**




| let-363 | | daf-15 | | rsks-1 | | sgk-1 | | skn-1 | |
|---|---|---|---|---|---|---|---|---|---|
| 0.01 mg/L | 0.1 mg/L | 0.01 mg/L | 0.1 mg/L | 0.01 mg/L | 0.1 mg/L | 0.01 mg/L | 0.1 mg/L | 0.01 mg/L | 0.1 mg/L |
| -4.7 | -4.7 | 2.5 | 3.7 | -4.7 | -7.2 | 1.8 | 2.0 | -1.5 | -1.6 |

(A) TCS. (B) TCC. Error bars represent standard error. "*" indicates significant difference from controls ($p<0.05$). Both TCS and TCC significantly decreased the expression of *rsks-1*, but TCC decreased the expression by a greater extent than TCS. TCC exposure significantly increased expression of *daf-15* and *sgk-1*, while significantly decreasing the expression of *let-363*.

with IGF and is involved in growth and reproduction (Korta et al., 2012). The examination of *let-363*, *rsks-1*, *sgk-1*, *daf-15*, and *skn-1* allow for the determination of the role of IGF in TCS- and TCC-induced toxicity.

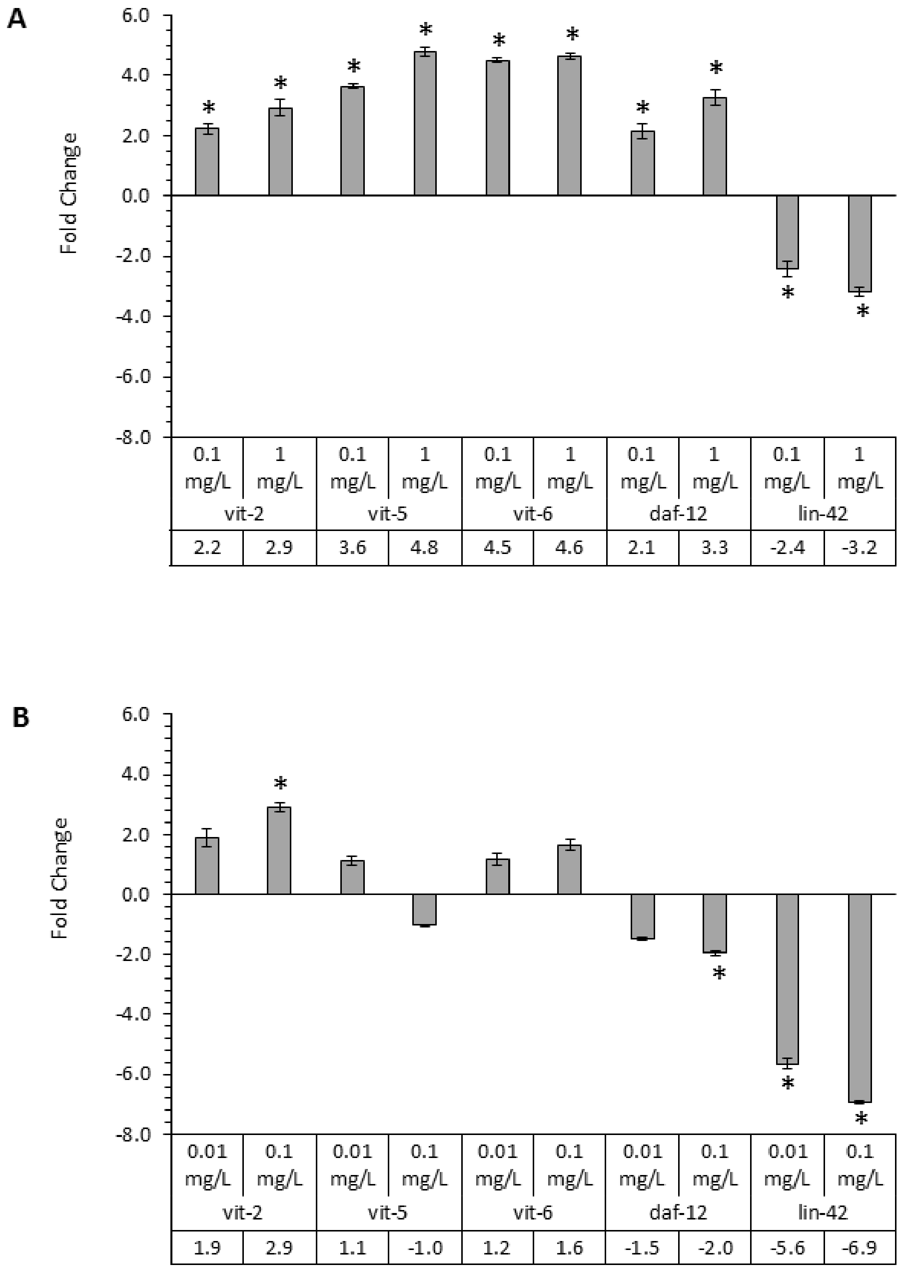
TCS and TCC caused varying effects on the expression of *let-363*, *rsks-1*, *sgk-1*, *daf-15*, and *skn-1* (Fig. 4.6). TCC exposure (0.1 mg/L) was also associated with decreased *let-363* and *rsks-1* expression by 4.7 and 7.2 fold respectively, while increasing *daf-15* and *sgk-1* expression by 3.7 and 2.0 fold, respectively (Fig. 4.6B). TCC exposure (0.1 mg/L) also decreased *rsks-1* by 7.2 fold, while TCS (1 mg/L) decreased *rsks-1* expression by less of an extant (2.6 fold) (Fig. 4.6). Unlike TCC, TCS didn't significantly impact the expression of *let-363*, *daf-15*, and *sgk-1*. TCS and TCC did not significantly impact the expression of *skn-1* at the observed concentrations.

*Development*

TCS and TCC cause delayed hatching, germline toxicity, and decreased reproduction in C. elegans (Lenz et al., 2017). In addition, our analysis of TCS and TCC exposure in IGF mutants resulted in decreased reproduction, decreased lifespan, and the development of avids, an age-related vulval integrity defect. The avid phenotype is regulated by the germline and hypothesized to be the result of reproduction causing the vulva to be a site of weakness that is exacerbated by the failure to shut down the production of yolk, protein, and fat (Leiser et al., 2016). Lipid redistribution, intestinal atrophy, and yolk synthesis are influenced by IGF signaling and vitellogenesis, as well as other genes involved in development (Ezcurra et al., 2018; Sornda et al., 2019). Therefore, we examined the impact of TCS and TCC exposure on the expression of vitellogenin (*vit-2*, *-5*, and *-6*), period protein homolog (*lin-42*), nuclear hormone receptor (*daf-12*) during early adulthood.

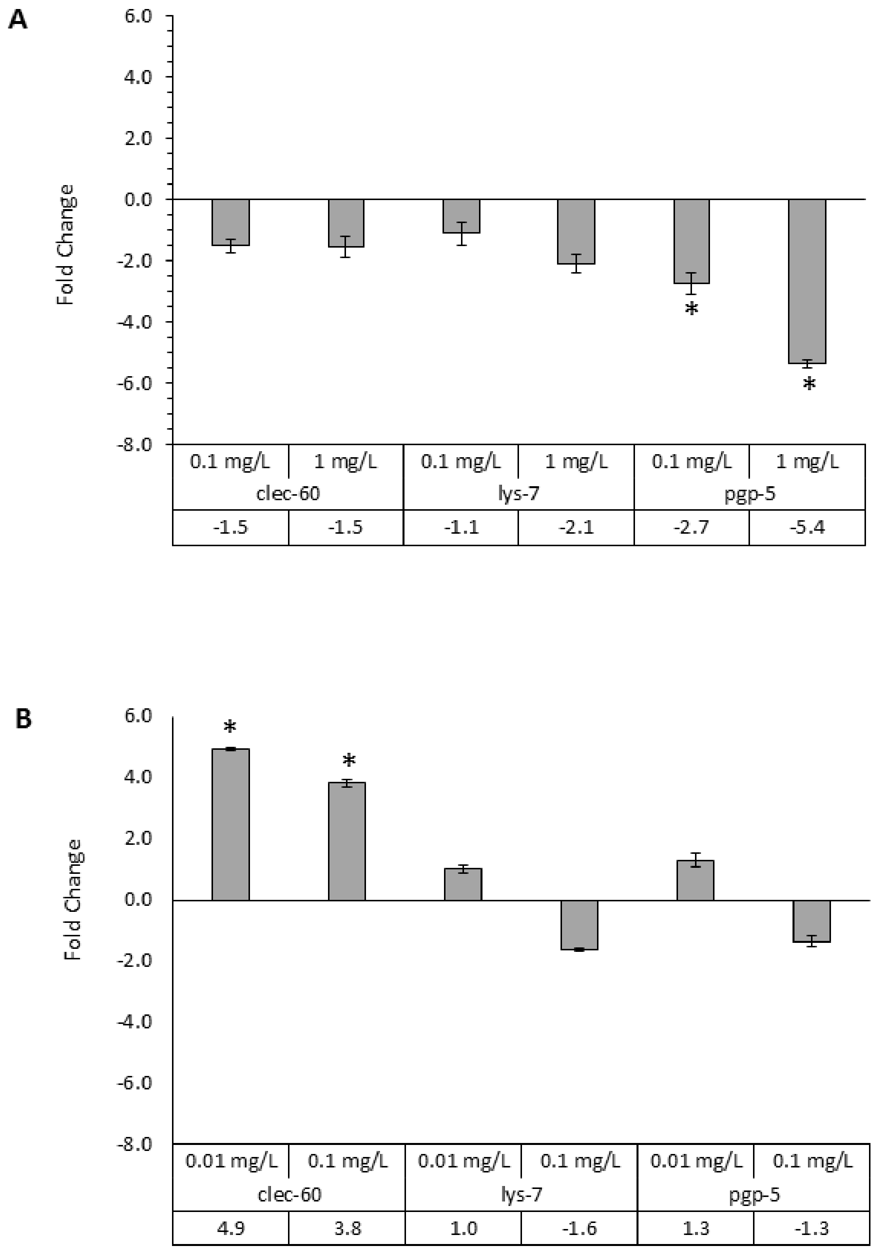
We found that in contrast to the gene expression associated with development and longevity genes controlling only development were impacted more by TCS compared to TCC (Fig. 4.7). TCC

Figure 4.7. Expression of developmental genes exposed to TCS and TCC.




(A) TCS. (B) TCC. “*” indicates significant difference from controls (p<0.05). Error bars represent standard error. TCS significantly increased the expression of three *vit* genes (2, 5, and 6) and *daf-12*, while TCC only significantly increased the expression of *vit-2*. Both TCS and TCC significantly decreased the expression of *lin-42*.

Figure 4.8. Expression of bacterial response genes exposed to TCS and TCC.




(A) TCS. (B) TCC. Error bars represent standard error. "*" indicates significant difference from controls ($p<0.05$). TCS significantly decreased the expression of *pgp-5*, while TCC significantly increased the expression of *clec-60*.

exposure (0.1 mg/L) was associated with increased *vit-2* expression by 2.9 fold and decreased *daf-12* and *lin-42* expression by 2.0 and 6.9 fold, respectively. TCC exposure did not significantly impact the expression of *vit-5* and *vit-6*. In contrast, TCS exposure increased *vit-2*, *vit-5*, *vit-6*, and *daf-12* expression by 2.9, 4.8, 4.6, and 3.3 fold, respectively, at 1 mg/L. TCS also decreased the expression of *lin-42* by 3.2 fold at 1 mg/L (Fig. 4.7). Both TCS and TCC increased the expression of *vit-2,* by approximately 3 fold. Increased expression of *vit-2* is associated with increased intestinal lipid content (Ezcurra et al., 2018).

*Bacterial response*

Lastly, TCS and TCC are used for their antimicrobial properties and there is concern that antimicrobials might induce antibiotic resistance or disrupt human microbial communities (Carey & McNamara, 2014). There was so much concern that in 2016 the Food and Drug Administration banned the use of of TCS and TCC in specific household products (FDA, 2016). A recent study found that TCS and TCC increased gut bacterial genes in mice and were associated with TCS resistance, stress response, and antibiotic resistance (Gao et al., 2017). In addition, a study of TCS and TCC exposure in infants found an association of increased presence of Proteobacteria species with TCS and TCC exposure (Ribado et al., 2017). Thus, we examined the effects of TCS and TCC on the bacterial response genes in *C. elegans*.

The examination of C-type Lectin (*clec-60*), lysozyme-like protein-7 (*lys-7*), and p-GlycoProtein-5 (*pgp-5*) found that TCS and TCC had different effects on these three genes. TCS exposure (1 mg/L) decreased the expression of *pgp-5* by 5.4 fold, but did not significantly decrease the expression of *clec-60* and *lys-7* (Fig. 4.8A). In contrast, TCC exposure (0.1 mg/L) increased the expression of *clec-60* by 3.8 fold at 0.1 mg/L and 4.9 fold (Fig. 4.8B). TCC exposure did not significantly impact the expression *lys-7* and *pgp-5*. TCS and TCC had a varying affect on genes involved in bacterial response. Only TCC increased the expression of one of the bacterial response genes, *clec-60*.

## Discussion

TCS and TCC are commonly used and widely detected in the environment and biological samples. Exposure to TCS and TCC have been associated with numerous adverse outcomes in wildlife and humans, such as developmental and reproductive toxicity, endocrine disruption, inhibition of muscle function, increased allergy sensitivity, and antibiotic resistance (Braoudaki and Hilton, 2004; Yazdankah et al., 2006; Chen et al., 2008; Cherednichenko et al., 2012; Savage et al., 2012; ECHA, 2015; EC, 2016). Several *in vitro, in vivo*, and epidemiological studies have found that endocrine disrupting abilities of these compounds include reduced production and/or metabolism of various hormones (i.e., testosterone, luteinizing hormone, follicle stimulating hormone, and estrogen), as well as the interacting with hormone receptors (Ahn et al., 2008; James et al., 2010; Huang et al., 2014; Henry and Fair, 2013; Kumar et al., 2009; Gee et al., 2008; Chen et al., 2008; Duleba et al., 2011; Stoker et al., 2010). More recently, the potential endocrine disrupting effects of TCS and TCC have been explored in invertebrates, such as insects, rotifers, and nematodes (Orvos et al., 2002; Han et al., 2016; Lenz et al., 2017; Martinez de Paz et al., 2017; Garcia-Espineira et al., 2018). In *C. elegans*, TCS and TCS have been reported to cause developmental and reproductive toxicity, decreased longevity, lipid accumulation, and the increased activation of daf-16/FOXO (Lenz et al., 2017; Garcia-Espineira et al., 2018). As the modulation of insulin/IGF signaling pathway is considered the central determinant of the worm's endocrine control of stress response and aging (Baumeister et al., 2006). We hypothesized that these antimicrobials may elicit toxicity in the worm through impacting the IGF pathway.

### Impact of TCS and TCC on gene expression

In *C. elegans*, IGF signaling is controlled by the activation of *daf-2* that initiates a signaling cascade involving *age-1* (phosphoinositide 3-kinase), *pdk-1* (phosphoinositide-dependent protein kinase-1) and *akt-1/2* (protein kinase B) (Fig. 1). The activation of this signaling cascade results in the phosphorylation of *daf-16* and inhibits its relocation into the nucleus (Murphy & Hu, 2005). Our study examined the expression of IGF genes in young adult worms (aged 3 days) and found that TCC exposure significantly increased *daf-2* expression by 3.1 fold at 0.1 mg/L, but not at 0.01 mg/L. In contrast, TCS did not increase the expression of *daf-2* (Figure 4.5). Both TCS and TCC were associated with increased expression of *age-1*, *pdk-1*, *akt-1*, and *akt-2* by >2 fold (Fig. 4.5). This increased expression of IGF genes downstream of *daf-2* by TCS and TCC suggests that these compounds may elicit toxicity through impacting the IGF signaling pathway. The activation of *daf-2*, increased expression of *daf-2*, by TCC is confirmed by the decreased expression of *daf-18* (>3 fold decrease) (Fig. 4.5B), as *daf-2* and *daf-18* have a negative regulatory relationship (Coogan & La Point, 2008). However, TCS does not exhibit the same negative regulatory relationship between *daf-2* and *daf-18* (Fig. 4.5A). TCS may not be inducing toxicity via *daf-2*, rather TCS may utilize its lipophilic properties (Log Kow 4.8) to bypass *daf-2* and initiate the IGF signaling cascade via genes downstream of *daf-2* (Coogan & La Point, 2008). TCC may also be able to bypass *daf-2*, which may explain the discrepancy between the the observed expression of *daf-2*, *pdk-1*, and *akt-1/2* (Fig. 4.5B). It is also possible that TCS may weaken the substrate-ligand interaction with the IGF receptor, such as what is exhibited by BαP (Fadiel et al., 2013). Additional research is needed to determine how TCS increases the expression of genes downstream of *daf-2*.

The increased expression of *age-1*, *pdk-1*, *akt-1*, and *akt-2* is consistent with the findings of increased *daf-2* expression and decreased *daf-18* expression, but these observations are not consistent with increased expression of *daf-16* (Ogg et al. 1998; Pierce et al., 2001; Palmitessa & Benovic, 2010; Liu et al., 2014). This increased *daf-16* expression by TCC exposure is accompanied by the decreased expression of *let-363* (target of rapamycin homolog) and *rsks-1* (ribosomal protein S6 kinase beta). The TORC1/Raptor (let-363/*daf-15*) complex is partially dependent upon the *daf-16* activity (Sun et al., 2017), which may explain the decreased *let-363* expression and increased *daf-15* expression (Figure 3B). However, the fact that the increased expression of *daf-16* is accompanied by the increased expression of

Table 4.7. Summary of sublethal endpoints in IGF mutants exposed to TCS

| Mutant Strain | Lethality | Reproduction | Lifespan | Avid Phenotype |
|---|---|---|---|---|
| *daf-2(e1368)* | Increased | Decreased | Similar | Similar |
| *ist-1(ok2706)* | Increased | Similar | Decreased | Increased |
| *age-1(hx546)* | Increased | Decreased | Decreased | Increased |
| *daf-18(ok480)* | Increased | Increased | Similar | Increased |
| *daf-18(e1375)* | Similar | Decreased | Increased | Increased |
| *pdk-1(sa680)* | Decreased | Decreased | Increased | Increased |
| *pdk-1(mg142)* | Increased | Decreased | Decreased | Increased |
| *akt-1(ok525)* | Increased | Decreased | Decreased | Increased |
| *akt-1(mg144)* | Increased | Decreased | Decreased | Increased |
| *akt-2(ok393)* | Increased | Decreased | Decreased | Increased |
| *daf-16(m26)* | Increased | Decreased | Decreased | Increased |
| *daf-16(m26); age-1(m333)* | Increased | Decreased | Decreased | Similar |
| *daf-16(m26); daf-2(e1370)* | Similar | Decreased | Decreased | Increased |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | Decreased | Decreased | Similar | Similar |
| *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* | Decreased | Decreased | Similar | Similar |

The involvement of IGF signalling in TCS-induced toxicity was examined in IGF mutant strains. Four endpoints were examined in *C. elegans*, such as 24 hour lethality, reproduction, lifespan, and an age-related vulval integrity defect, Avid. The above table is a summary of the data collected for each IGF mutant that was exposed to TCS. The summary was catergorized as increased, decreased, or similar toxicity compared to observations in wildtype worms exposed to TCS. Similar is defined as similar toxicity to wildtype worms exposed to TCS. Increased is increased toxicity compared to wildtype worms exposed to TCS. Decreased is defined as decreased toxicity compared to wildtype worms exposed to TCS.

Table 4.8. Summary of sublethal endpoints in IGF mutants exposed to TCC

| Mutant Strain | Lethality | Reproduction | Lifespan | Avid Phenotype |
|---|---|---|---|---|
| *daf-2(e1368)* | Decreased | Similar | Increased | Similar |
| *ist-1(ok2706)* | Similar | Decreased | Decreased | Increased |
| *age-1(hx546)* | Decreased | Decreased | Similar | Increased |
| *daf-18(ok480)* | Increased | Increased | Increased | Increased |
| *daf-18(e1375)* | Increased | Decreased | Increased | Increased |
| *pdk-1(sa680)* | Increased | Decreased | Increased | Similar |
| *pdk-1(mg142)* | Similar | Decreased | Similar | Increased |
| *akt-1(ok525)* | Increased | Decreased | Decreased | Increased |
| *akt-1(mg144)* | Increased | Increased | Decreased | Increased |
| *akt-2(ok393)* | Decreased | Decreased | Decreased | Similar |
| *daf-16(m26)* | Increased | Similar | Decreased | Increased |
| *daf-16(m26); age-1(m333)* | Decreased | Decreased | Decreased | Similar |
| *daf-16(m26); daf-2(e1370)* | Increased | Similar | Increased | Increased |
| *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* | Decreased | Decreased | Decreased | Similar |
| *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* | Decreased | Decreased | Decreased | Similar |

The involvement of IGF signalling in TCC-induced toxicity was examined in IGF mutant strains. Four endpoints were examined in *C. elegans*, such as 24 hour lethality, reproduction, lifespan, and an age-related vulval integrity defect, Avid. The above table is a summary of the data collected for each IGF mutant that was exposed to TCC. The summary was catergorized as increased, decreased, or similar toxicity compared to observations in wildtype worms exposed to TCC. Similar is defined as similar toxicity to wildtype worms exposed to TCC. Increased is increased toxicity compared to wildtype worms exposed to TCC. Decreased is defined as decreased toxicity compared to wildtype worms exposed to TCC.

*daf-2*, *age-1*, *pdk-1*, *akt-1*, and *akt-2* suggests that there are other genes and/or pathways influencing *daf-16* expression. Two explanations for the observed increased *daf-16* expression are (1) *ist-1* is influencing *daf-16* activity by acting in parallel to *age-1* or (2) *daf-16* activity is caused by other signaling pathways (i.e., germline signaling, JNK, and AMPK) (Murphy & Hu, 2005).

**Toxicity in mutant strains**

We examined the role of IGF signaling in the observed TCS- and TCC-induced toxicity. The IGF signaling pathway, or insulin-like growth pathway, has an important role in endocrine function due to its regulation of several processes, such as aging, reproduction, stress response, and development (Murphy & Hu, 2005; Baumeister et al., 2006; Anisimov & Bartke, 2013). Studies in *C. elegans* found that TCS and TCC exposure were associated with developmental and reproductive toxicity, decreased longevity, lipid accumulation, and altered activity of genes involved in the worm's stress response (Lenz et al., 2017; Garcia-Espineira et al., 2018). This suggests the potential involvement of IGF signaling in the observed toxicity. Thus, we performed the genetic analysis of TCS- and TCC-induced toxicity using IGF mutants. The results showed TCS and TCC had varying effects on lethality, reproduction, lifespan, and avid phenotype that were dependent upon the gene mutation (Table 4.7 and 4.8).

*Impact of TCS and TCC in IGF mutants*

Increased lethality was a more accurate indicator of the effects of TCS and TCC exposure on avid phenotype than reproduction and lifespan. For example, the functional loss of *daf-2* and *age-1* exhibited a protective effect in *daf-2(e1368)*, *age-1(hx546)*, and *daf-16(m26); age-1(m333)* worms exposed to TCC (Tables 4.2 and 4.6). However, *daf-2(e1368)* worms were more sensitive to TCS (LC50 0.7 mg/L) than wildtype worms (LC50 3.7 mg/L) (Table 4.2). Meanwhile, a functional loss of *pdk-1* was protective in *pdk-1(sa680)* worms exposed to TCS (Tables 4.2 and 4.6), as well as a functional loss of *daf-2* in *daf-16(m26); daf-2(e1370)* worms (Table 4.2). This suggests that TCS- and TCC-induced acute toxicity is potentially mediated by genes upstream of *daf-16*, which is supported by the increased acute toxicity seen in worms with the loss-of-function of *daf-16* in *daf-16(m26)* worms (Table 4.2), as well as supported by the changes in the expression of *age-1*, *pdk-1*, and *akt-1/2* by both TCS and TCC (Fig. 4.5). However, the involvement of IGF genes in the observed toxicity varies between TCS and TCC exposure.

*Impact of TCS and TCC on age-1, pdk-1,* and *akt-1*

The involvement of *age-1, pdk-1,* and *akt-1* in TCS- and TCC-induced toxicity seen by the significantly increased gene expression is further supported by the decreased toxicity of both TCS and TCC in *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* and sqt-1(sc13) age-1(mg109) II; akt-1(mg247) worms. Both TCS and TCC were less toxic in *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms, indicated by decreased acute lethality, reproductive toxicity, and avid phenotypes. The LC50s of TCS in *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms were 5.8 (95% CI: 5.3, 6.3) and 6.6 (95% CI: 5.9, 7.2), respectively, compared to 3.7 (95% CI: 3.2, 4.3) in wildtype worms. Meanwhile, the LC50s of TCC in *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms were 2.5 (95% CI: 2.0, 3.1) and 8.9 (95% CI: 7.8, 9.6), respectively, compared to 0.9 (95% CI: 0.5, 1.5) in wildtype worms (Table 4.2). The significance of these findings in *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* and *sqt-1(sc13) age-1(mg109) II; akt-1(mg247)* worms is due to the genetics of these two mutants.

The pairing of *age-1(mg109),* constitutive dauer arrest (*daf-C*) phenotype, with a suppressor of *daf-C*, *pdk-1(mg261)* or *akt-1(mg247),* gain-of-function of phosphoinositide-dependent serine/threonine kinases, *pdk-1* and *akt-1*, are known to only partially restore IGF signaling (Gami et al., 2006). This is due to the fact that *sqt-1(sc13) age-1(mg109); pdk-1(mg261)* worms require functional *akt-1,* while *sqt-1(sc13) age-1(mg109); akt-1(mg247)* worms require functional *pdk-1* (Gami et al., 2006). The requirement of functional *pdk-1* and *akt-1* by these worms to restore IGF functioning and reproductive development may explain our data in two ways. First, the *mg247* and *mg261* alleles only partially restores phospholipid signaling in *age-1(mg109)* worms (Gami et al., 2006), preventing the overactivation of IGF and allowing *daf-16* to activate and respond the stressors, TCS and TCC. This is supported by the decreased toxicity observed in *akt-1(mg247)* and *pdk-1(mg261)* worms, which are known to not affect lifespan and stress resistance (Gottlieb & Ruvkun, 1994; Gems et al., 1998; Wolkow et al., 2002). Second, TCS- and TCC-induced toxicity may act through a specific *akt-1* allele. Gami and colleagues (2006) reported that *sqt-1(sc13) age-1(mg109); akt-1(mg227)* worms only require *akt-1* for reproductive development and *pdk-1* activity was dispensable, but *sqt-1(sc13) age-1(mg109); akt-1(mg247)* require the presence of *pdk-1* for reproductive development. Further examination is needed to determine if the toxicity of TCS and TCC is dependent on the relationship of *akt-1 and pdk-1* or is *akt-1* allele-dependent, as there are 40 identified *akt-1* alleles known to suppress daf-C (Gami et al., 2006).

*Impact of TCS and TCC on daf-18*

The findings from *sqt-1(sc13) age-1(mg109); pdk-1(mg261) and sqt-1(sc13) age-1(mg109); akt-1(mg247)* worms are also supported by the increased acute toxicity, decreased lifespan, and avid phenotypes of *daf-18(ok480)* worms. TCS and TCC were most toxic in *daf-18(ok480)* worms, a strain with the severe loss-of-function of *daf-18* (Fukuyama et al., 2006), which in wildtype worms counteracts *age-1* activation through the dephosphorylation of *pip3* (Figure 1). The *daf-18(ok480)* worms were associated with increased acute lethality, reproductive toxicity, and avid phenotype. The LC50 of TCS and TCC was 0.2 (95% CI: 0.1, 0.4) and 0.008 (95% CI: 0.005, 0.01) in *daf-18(ok480)* worms compared to 3.7 (95% CI: 3.2, 4.3) and 0.9 (95% CI: 0.5, 1.5) in wildtype worms, respectively (Table 2). Additionally, TCC exposure was associated with increased acute lethality in *daf-18(e1375)* worms [0.2 (95% CI: 0.1, 0.3)], a reduction-of-function strain (Ogg & Ruvkun, 1998). The increased acute lethal toxicity observed in *daf-18(ok480)* and *daf-18(e1375)* worms suggests that potential increased expression of *daf-2.* This is confirmed by the the fact *daf-2* and *daf-18* have a negative regulatory relationship, meaning the decreased expression of *daf-18* is associated with increased expression of *daf-2* (Liu et al., 2014).

*Development of an age-related vulval integrity defect (Avid)*

In addition to increased acute lethality, the *ok480* allele of *daf-18* is reported to cause a mitotic germline arrest defect, associated with increased mortality that was previously reported in this mutant (Fukuyama et al., 2006). In our study, we observed an increased frequency of avid phenotype in post-reproductive *daf-18(e1375)* and *daf-18(ok480)* worms exposed to TCS and TCC, as well as controls (Table 4.6). Both TCS and TCC were associated with increased frequency of avids in *ist-1(ok2706), age-1(hx546), daf-18(ok480), pdk-1(mg142), akt-1(ok525), akt-1(mg144), akt-2(ok393), daf-16(m26),* and *daf-16(m26); age-1(m333)* worms. Furthermore, an increased frequency of avids occurred in *ist-1(ok2706), daf-18(ok480), daf-18(e1375),* and *akt-1(mg144)* controls, at >20% in control worms (Table 4.6). This increased frequency of avids in controls was associated with a significantly decreased lifespan (average lifespan ranging from 11.8 to 14.4 days) compared to wildtype controls [17.2 days (CI 95%: 17.0, 17.3)] (Tables 4.4 and 4.5). The development of an avid phenotype is previously reported in the literature for both *daf-18* mutants, *daf-18(ok480)* and *daf-18(e1375),* at rates up to 80% (Ogg & Ruvkun, 1998; Fukuyama et al., 2006). We observed an increased frequency of avids in *daf-18(ok480)* and *daf-18(e1375)* worms reaching 68% compared to <10% in wildtype worms (Table 4.6). Ogg and colleagues (1998) also reported an avid phenotype in wildtype worms with inhibited *daf-18* activity, suggesting the importance of *daf-18* activity in the regulation of healthspan.

Avid phenotypes are known to be modulated by the germline and are associated with decreased lifespan and a loss of oocyte production, but not sperm production (Leiser et al., 2016). Leiser and colleagues (2016) hypothesize that avids are the result of reproduction causing the vulva to be a site of weakness that is exacerbated by the failure to shut down the production of yolk, protein, and fat. In our study, we found that mutants with decreased longevity in controls were associated with increased frequency of an avid phenotype and decreased reproduction. These increased avid phenotypes occurred at higher rates in *ist-1(ok2706), daf-18(ok480),* and *daf-18(e1375)* controls (Table 4.6) and was associated with a significantly decreased lifespan (average lifespan ranging from 11.8 to 14.4 days) compared to wildtype controls [17.2 days (CI 95%: 17.0, 17.3)] (Table 4 and 5). In *daf-18(ok480)* worms, TCS and TCC decreased reproduction by >50% compared to controls (Table 4.3). This decrease in reproduction was associated with a high frequency of vulval integrity defect in worms (Table 4.6) and decreased lifespan (Table 4.4). However, this effect was not seen in worms with the loss of functional *daf-2, age-1, pdk-1, akt-1/2,* and *daf-16*. This is supported by the fact that under normal conditions *daf-18* regulates the germline checkpoint that opposes the proliferation and growth-promoting activities of *age-1* and *akt-1* (Paradis & Ruvkun, 1998), and suggests the potential involvement of IGF activity in the development of avid phenotypes.

It is hypothesized that the overactivation of IGF causes the development of an avid phenotype and decreased lifespan. Ezcurra and colleagues (2018) found that lipid redistribution, intestinal atrophy, and yolk synthesis was *daf-2* and *daf-16* dependent, supporting our hypothesis. Additionally, the inhibition of yolk proteins production by *vit-5/6* was associated with decreased intestinal lipid content, while the inhibition of yolk protein production by *vit-2* was associated with increased intestinal lipid content (Ezcurra et al., 2018). We found that in young adult, wildtype worms (aged 3 days) exposed for 24-hours both TCS and TCC increased the expression of *vit-2*, while TCS also increased the expression of *vit-5* and *vit-6* (Fig. 4.7). We did not examine the expression of *vit* genes in the mutants with increased frequency of avid phenotype. However, increased levels of *vit* mRNA in early adulthood worms in low fertility *daf-2(e1370)* hermaphrodites has been reported (DePina et al., 2011). We hypothesize that the overactivation of IGF and increased expression of specific *vit* genes may be contributing to increased yolk proteins production and intestinal lipid content that may promote the development of an avid phenotype. If this is the case, this would support the hypothesis that the avid phenotype is analogous to obesity in higher organisms (Leiser et al., 2016), which is an adverse outcome associated with the overactivation of IGF, decreased lifespan, and decreased reproduction but not sperm production (Seidell et al., 1990; Berryman et al., 2013; Lewitt et al., 2014). Additional research is needed to determine the role of IGF and vitellogenesis in the development of an avid phenotype.

**Conclusions**

TCS and TCC are known endocrine disruptors that have been associated with increased obesity risk, reduced birthweight, infertility, decreased lifespan, and development and proliferation of cancer cells (Halden et al., 2017). Previous research showed that TCS and TCC decreased lifespan and reproduction, as well as increased *daf-16* activity, suggesting the involvement of IGF in TCS- and TCC-induced toxicity. To the best of our knowledge this is the first study to examine the effects of TCS and TCC on the IGF signaling pathway. We found that TCS- and TCC-induced toxicity was suppressed by loss-of-function mutants and increased in mutants with overactivated IGF, which was supported by gene expression data. The data collected supports the role of IGF signaling in TCS- and TCC-induced toxicity; however it is not clear if TCS and TCC activate this signaling cascade through *daf-2*. The lipophilic nature of both TCS and TCC may allow for the bypass of *daf-2*. Additional research is needed to determine how TCS and TCC alter the expression of IGF, as well as role of IGF and vitellogenesis in the development of avids.

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CHAPTER 5:

# The toxicological effects of TCS, TCC, and their degradation products in the nematode *C. elegans*

## Abstract

Triclosan (TCS) and triclocarban (TCC) are antimicrobials that enter the environment everyday through the use of personal care, consumer, and medical products. Once in the environment, TCS and TCC are exposed to solar ultraviolet (UV) radiation that can degrade these compounds and result in the formation of toxic and carcinogenic products, such as dioxins, furans, chlorophenols, and anilines. These compounds have been widely detected in the environment including surface water, soil, and sediment. In 2017, *The Florence Statement on TCS and TCC* highlighted the need to evaluate the safety of TCS, TCC, and their transformation products throughout the entire product life cycle, including environmental release. Here we assessed the potential toxicological effects of TCS, TCC, and their photodegradation products to a model organism the nematode *Caenorhabditis elegans* (*C. elegans*). We examined the toxicological effects of parent and UV exposed TCS and TCC using organismal and molecular endpoints, including lethality, reproduction, hatching time, growth rate, and lifespan. TCS and TCC were toxic at low mg/L levels, and TCC was more toxic than TCS. The UV exposure of TCS and TCC resulted in increased toxicity of both compounds. The 24 hour lethality of 168 hours of UV exposure to TCS and TCC decreased from 3.65 mg/L to 0.81 mg/L and 0.91 mg/L to 0.04 mg/L, respectively. At sublethal concentrations, TCS and TCC exposure decreased the total number of offspring produced by worms. Furthermore, UV exposure of TCS (0.1 mg/L) and TCC (0.01 mg/L) decreased reproduction in the worms, indicated by a decreased brood size per worm from 29% to 46% and 32% to 55%, respectively. UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) delayed hatching and decreased the growth rate of larval worms. Additionally, Lifespan was decreased on average by 3.7 days for parent TCS (0.1 mg/L) and TCC

(0.01 mg/L) and 5.1 and 4.5 days, days by UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L). These findings suggest that TCS and TCC may pose significant health risks to aquatic organisms. Our next steps are to characterize the degradation products that were formed during the UV exposure and understand the underlying mechanism(s) of observed reproductive toxic effects of TCS, TCC, and their degradation products.

**Introduction**

Triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol; TCS] and triclocarban [trichlorocarbanilide, 3-(4-chlorophenyl)- 1-(3,4-dichlorphenyl)urea; TCC] are antimicrobial agents used in >2,000 personal care and consumer products that are washed down the drain and end up in the environment every day (Aranami et al., 2007; Halden et al., 2017). TCS and TCC are introduced into aquatic environments through aquaculture, agriculture, animal husbandry, pharmaceutical companies, wastewater and sewage treatment plants, hospitals, and household discharge (Kanama et al., 2018). Once in the environment, TCS and TCC persist in the environment for decades, with half-lives of 60 days in water, 120 days in soil, and 540 days in sediment (Halden & Paull, 2005). Furthermore, the long-term preservation of TCS and TCC has been shown in sediment cores dating back to 1964, when TCS was patented (Singer et al., 2002; Miller et al., 2008; Cantwell et al., 2010; Bedoux et al., 2012; Anger et al., 2013; Kerrigan et al., 2015). In boisolids-amended soils, TCS and TCC have shown to persist for extended period of time while exhibiting very slow or no measurable degradation due to their chemical stability and pH resistance. (Walters et al., 2010; Langdon et al., 2012; Halden, 2016).

The environmental persistence of TCS and TCC is attributed to their chemical properties. TCS and TCC have similar water solubility, ranging from 1.97-4.6 and 0.65-1.55, respectively, and partitioning coefficients (4.8 and 4.9, respectively) similar to bisphenol A (BPA), polychlorinated biphenyls (PCBs), and dioxins (Halden, 2014; Dhillon et al., 2015). In 1993, TCS was labelled as a pre-dioxin by the US

Environmental Protection Agency (EPA) due to its chemical properties and transformation into known toxic and carcinogenic compounds (Halden, 2014). TCS and TCC are the source of toxic and carcinogenic products (i.e., dioxins, furans, chlorophenols, and anilines) that can be formed during the manufacturing process, aerobic biodegradation, photolysis, methylation, and chlorination (Fiss et al., 2007; Buth et al., 2010; Ding et al., 2013). TCS is known to contain detectable levels of 2,3,7,8-tetrachlorodibenzop-dioxin and 2,3,7,8-tetrachlorodibenzofuran during the production products, levels varying based on the quality of production technology (Menoutis and Parisi, 2002; Zheng et al., 2008; IARC, 2012; UNEP, 2013). Similarly, toxic byproducts are detected in TCC following production, such as 4-chloroaniline and 3,4-dichloroaniline, as well as as well as tetrachlorinated TCC (Eissa et al., 2012; Halden, 2014).

Transformation of TCS and TCC can occur during wastewater and drinking water treatment and in the environment (Lazano et al., 2013). Both TCS and TCC are degraded via methylation and dechlorination, respectively, during sewage conveyance and treatment and anaerobic conditions (Pycke et al., 2014). Once in the environment, TCS and TCC can be degraded further into different polychlorinated compounds. TCC undergoes aerobic biodegradation and photodegradation into 3,4-dichloroaniline, 4-chloroaniline and 4-chlorocatechol (Miller et al., 2010; Ding et al., 2013; Mulla et al., 2016). TCS is converted to 2,8-dibenzodichloro-*p*-dioxin (2,8-DCDD) during heating and combustion (Kanetoshi et al., 1988) and when exposed to natural sunlight (Latch et al., 2003; Aranami and Readman, 2007; Alvarez-Rivera et al., 2016). Furthermore, the chlorinated derivatives of TCS, such as DCDD, can transform into tri- and tetra-chlorinated dibenzo-*p*-dioxins with continued exposure to sunlight (Buth et al., 2009; Buth et al., 2010). TCS can also be methylated, glycosylated and glucuronidated (James et al., 2012; Weatherly et al., 2017).

In recent years, several studies have been conducted on the toxicological effects of TCS and TCC. However, little is known about the safety of TCS and TCC transformation products throughout the entire product life cycle of the chemicals (e.g., manufacture, long-term use, disposal, and environmental release), leading to the publication and signing of a consensus, known as *The Florence Statement on TCS and TCC*, signed by over 200 experts (Halden et al., 2017). TCS and TCC are detected in a wide variety of matrices worldwide, including blood, urine, breast milk, aquatic organisms, various crops, sediments, and soils (Aranami et al., 2007). Several studies have demonstrated the occurrence and fate of these contaminants across the globe have recently been detected in house dust, ocean water, and the water loop of spacecraft (Allmyr et al. 2008; Halden et al., 2014). During wastewater treatment, TCS and TCC accumulate in sewage sludge, 30–70% and 70-90%, respectively, and the remaining TCS and TCC is discharged from the wastetwater treatment plant into the receiving stream where they partition into sediment and/or bioaccumulate in biota (Pycke et al., 2014). Once in the environment, TCS and TCC are photooxidized and photolysed to form additional degradation products, some of which are toxic and carcinogenic (Lehutso et al., 2017). Photooxidation occurs when the oxygen radicals disrupt covalent bonds where the oxygen radicals are produced from the interaction of UV with organic matter (Suarez et al., 2007; Buth et al., 2010; Weatherly et al., 2017). Photolysis is direct absorption of light by TCC/TCS at wavelengths that disrupt covalent bonds (Trouts et al., 2015; Weatherly et al., 2017). A few studies have examined the photofate of TCS and TCC using both artificial UV light and natural sunlight (Cawley et al., 2009; Guerard et al., 2009; Wenk et al., 2011; Wenk et al., 2012; Ding et al., 2013; Ding et al., 2014). These studies have only examined the acute toxicity of photodegradated TCS and TCC, but not the sublethal toxicity of these degradation products in mixture.

In contrast to the abundant literature on parent TCS and TCC, there is little toxicological information available on the toxicity of the environmental degradation products of TCS and TCC, as mixtures. Given this lack of knowledge regarding the toxicity of the environmental degradation products, an excellent starting point would be to screen for the potential toxicity using an ecological relevant model, *Caenorhabditis elegans* (*C. elegans*) (Leung et al., 2008). *C. elegans* is a free-living nematode found in soil, sediment, and freshwater, environments where TCS and TCC have been detected. Nematodes are  a suitable model organism for toxicological studies because of the availability of genetic tools, well-understood biology, transparent bodies, conserved molecular and cellular pathways and ease of culture maintenance or breeding (Hoss and Weltie, 2007; Leung et al. 2008; Haegerbaeumer et al., 2018). Here, we compared the toxicological effects of TCS, TCC, and their environmental degradation products using a model organism the nematode *C. elegans*. We examined the toxicological effects of TCS, TCC, and their environmental degradation products using organismal and molecular endpoints, including lethality, hatching rate, growth rate, reproduction, and lifespan. Findings from this study will provide a foundation for the examination of the potential impact of the degradation of TCS and TCC on their toxicity. This will address current knowledge gaps and provide an understanding of the environmental and human health implications of the environmental degradation products of TCS and TCC.

**Methods**

*Chemicals*

TCS (≥97% purity) and TCC (≥98.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions (1,000 mg/L dissolved in dimethyl sulfoxide) were used to create all the exposure concentrations with K-medium (51 mM NaCl, 32 mM KCl, pH 6.8) as the diluent. TCS exposure concentrations were 0.01 to 10 mg/L. TCC exposure concentrations were 0.001 to 10 mg/L. All sub-lethal concentrations were the same as our previous study (Lenz et al., 2017). The final DMSO concentration was <1% for acute lethality and < 0.5% for sub-lethal exposures.

*Strains*

All *C. elegans* strains used in this study, the N2 Bristol (wildtype), were obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). All nematodes were maintained on nematode growth medium agar plates seeded with *E. coli* OP50 maintained at 20°C (Stiernagle, 2006). Age-synchronized populations were obtained for acute lethality and sub-lethal assays through the collection of eggs from gravid adult *C. elegans*. The cleaning of eggs was done by bleaching with 1% NaClO and 0.013M NaOH solution and followed by a K-medium wash (Donkin & Williams, 1995).

*UV Exposure*

UV exposures were conducted to determine if the degradation of TCS and TCC by ultraviolet (UV) radiation impacted the toxilogical effects observed in *C. elegans*. We conducted our UV exposures using a Blak-Ray UV Benchtop Lamp (Ultra-Violet Products Ltd, Upland, CA) to expose TCS and TCC using UVA (365 nm), the wavelength that accounts for approximately 95% of the UV radiation reaching Earth's surface (WHO, 2019). UV exposures were conducted for 24, 72, 120, 168 hours for both TCS and TCC. As a control, a second set of exposures of TCS and TCC were exposed at room temperature on the lab bench top for 24, 72, 120, and 168 hours. The UV and ambient exposures were reported as "UV" and "parent" TCS and TCC. Furthermore, comparisons with data from the literature refer to TCS and TCC toxicity exposures that are not exposure to room temperature or UV. This data is referred to as "unexposed" TCS and TCC. All acute toxicity assays used exposed TCS and TCC at all 4 times points, while sublethal assays use TCS and TCC exposed for 168 hours.

*Lethality*

Lethality assays were previously described by Lenz and colleagues (2017). Young adult, age-synchronized worms (aged 3 days) were placed in 24-well plates containing 1 mL of exposure solutions made with K-medium. Each exposure consisted of 3 wells containing 10 worms each with an exposure time lasting 24 hours. The exposures consisted of K-medium (negative control), TCS (0.01 to 10 mg/L), or TCC (0.001 mg/L to 10 mg/L) and excluded any feeding agent. After 24 hours, the mortality was scored using a dissecting microscope. The LC50s were calculated using logistic regression of exposure and percent mortality using SigmaPlot (Systat Software, Inc., San Jose, CA).

*Reproduction*

Age-synchronized worms, aged 2 days (L4-staged), were placed on OP50 seeded NGM plates amended with 150 µL of K-medium (negative control), TCS (0.1 and 1 mg/L), or TCC (0.01 mg/L and 0.1 mg/L). NGM exposure plate preparation for exposures was previously described by Lenz and colleagues (2017). Each plate contained one L4-staged worm that was stored at 20°C for the duration of the exposure. Every other day the parent worm was transferred to a fresh plate (a total of 2-3 transfers over approximately 6 days) and the number of progeny (eggs and larval worms) was recorded. The average number of progeny was calculated by adding the number of progeny from all plates per worm (a total of 3-4 plates per worm) for all exposures.

*Hatching and growth rate*

The experimental methods for hatching time, the time from egg disposition to hatching (Muschiol et al., 2009), and growth rate, the size (body length) of worms, have been previously described by Lenz et al. (2018). For the hatching time assay, eggs from age-synchronized young adult worms (aged 3 days) were collected and prepared using a standard protocol (Donkin and Williams, 1995). Approximately 300 eggs were placed on an OP50 plate amended with 300 mL of K-medium (negative control), TCS (0.1 and 1 mg/L), and TCC (0.01 and 0.1 mg/L). Every 3 hours, the total number of hatched eggs, L1 staged worms, were recorded. Following the hatching of all worms, the larval worms were examined for growth.

For the growth assay, the body length, length from the opening of the mouth (anterior end) to the tip of the tail (posterior end) (Morck and Pilon, 2006), of larval worms were measured at 4 time points (18, 24, 48, and 72 hours) during the four larval stages (L1-L4) of development for *C. elegans*. Byerly (1976) has extensively described the growth of wildtype worms. Images of the worms were taken on NGM plates for each exposure condition using a Nikon AZ100 microscope and NIS-Elements BR 3.2

software and analyzed for body length measurement of all worms using Zen 2011 software (Carl Zeiss Microscopy). Approximately 100 worms were measured at each time point for each exposure condition and the average body length was calculated.

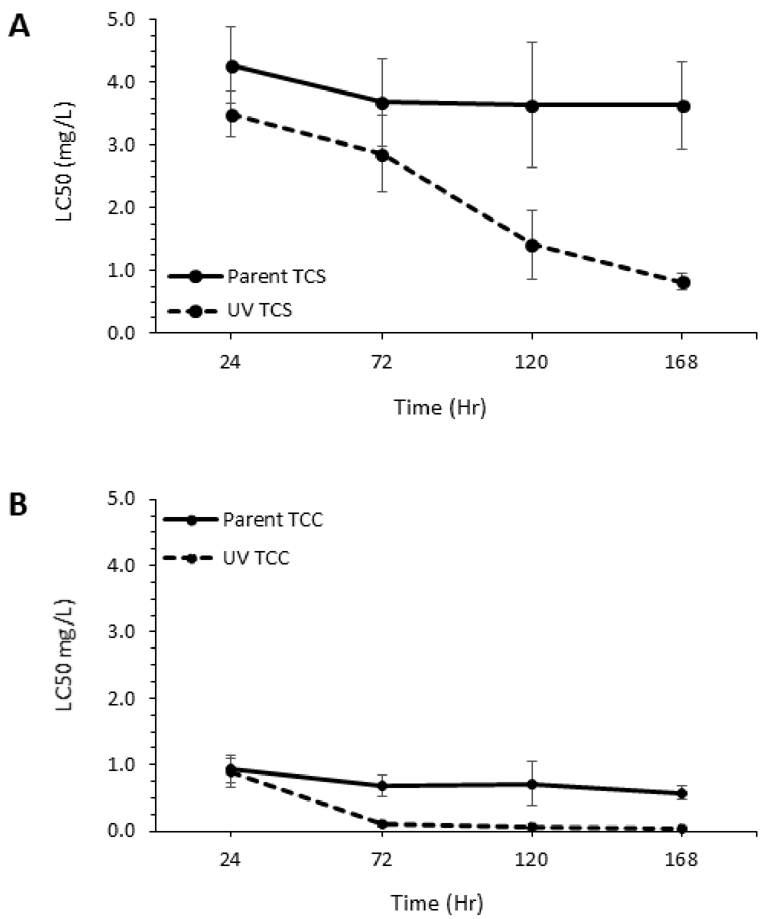
*Lifespan*

Lifespan experiments followed the standard protocol, exposing L4-staged worms on NGM plates seeded with OP50 and 0.2 mM 5-fluoro 2-deoxyuridine (FUdR) to inhibit progeny production (Pluskota et al., 2009). Similar to reproduction, plates seeded with OP50 and FUdR were amended with 150 µL of K-medium (negative control), TCS (0.1 and 1 mg/L), or TCC (0.01 mg/L and 0.1 mg/L). Two plates containing 50 worms each were used for each exposure (100 total worms per exposure per replicate). Every other day dead worms, defined as the failed response to an external stimulus applied to the anterior and posterior ends with a platinum picker, were removed and counted per exposure plate and recorded. Any burrowed worms were excluded from totals. This process occurred for approximately 3 weeks.

*Data analysis*

All assays were repeated three times. One-way analysis of variance was performed for the comparison of differences between individual TCS and TCC exposures compared to controls, as well as between parent and UV exposed TCS and TCC. Lifespan was analyzed using Kaplan-Meier survival analysis, estimation of the mean lifespan, and log-rank test, comparison of survival function between individual exposures and between strains. Data analysis conducted using IBM SPSS Statistics (Armonk, NY).

Figure 5.1. Acute toxicity in young adult wildtype *C. elegans* following a 24-hour exposure to TCS, TCC, and their photodegradation products.




**C**

| | LC50 (mg/L) | | | |
|---|---|---|---|---|
| Length of Exposure (Hr) | Parent TCS | UV TCS | Parent TCC | UV TCC |
| 24 | 4.27 (3.68, 4.89) | 3.49 (3.13, 3.84) | 0.94 (0.74, 1.20) | 0.88 (0.67, 1.16) |
| 72 | 3.68 (2.97, 4.45) | 2.85 (2.24, 3.59) | 0.67 (0.51, 0.88) | 0.095 (0.064, 0.14) |
| 120 | 3.64 (2.63, 4.51) | 1.41 (0.87, 2.36) | 0.71 (0.39, 1.28) | 0.053 (0.042, 0.070) |
| 168 | 3.64 (2.93, 4.39) | 0.81 (0.68, 0.98) | 0.58 (0.47, 0.74) | 0.043 (0.034, 0.055) |

To evaluate the toxicity of these degradation products, we tested the toxicity of TCS and TCC exposed to ultraviolet (UV) radiation, specifically UVA (365 nm), for 24, 72, 120, and 168 hours. As a control, parent TCS and TCC are exposed at room temperature for 24, 72, 120, and 168 hours. (A) Change in LC50 of TCS following room temperature and UV exposures over 168 hours. (B) Change in LC50 of TCC following room temperature and UV exposures over 168 hours. (C) Comparison of LC50s of TCS, TCC, and their photodegradation products. The 24 hour lethality of unexposed TCS and TCC is 3.65 mg/L and 0.91 mg/L, respectively (Lenz et al., 2017). After 168 hours of UV exposure, TCS and TCC increased in toxicity, exhibited through a 24 hour lethality of 0.81 mg/L and 0.04 mg/L, respectively. The difference in LC50 of worms exposed to UV TCS and TCC compared to parent TCS and TCC was significant ($p<0.05$).

## Results

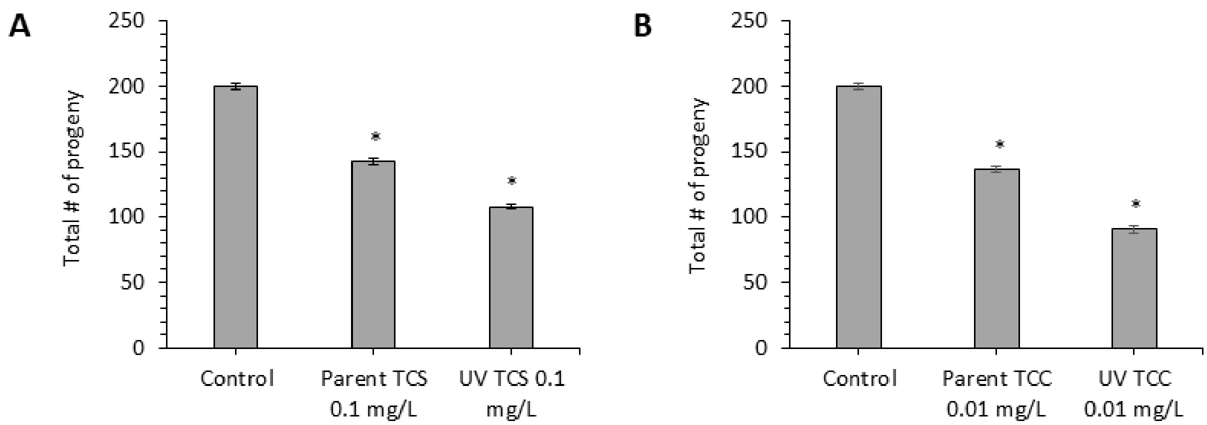
*Impact on 24 hour lethality*

We examined the effect of UV exposure (365 nm) on the 24-hour acute toxicity of TCS and TCC in young adult wildtype *C. elegans* at 4 times points (24, 72, 120, and 168 hours). As a control, parent TCS and TCC are exposed at room temperature for 24, 72, 120, and 168 hours. We found that both TCS and TCC caused acute toxicity to *C. elegans* at low mg/L concentrations. TCC is more toxic than TCS, which is consistent with our previous study (Lenz et al., 2017). Unexposed TCS and TCC had LC50s of 3.65 mg/L [95% Confidence Interval (CI): 3.15, 4.3] and 0.91 mg/L [95% CI: 0.47, 1.53] (Lenz et al., 2017). After a 24 hour exposure to UVA (365 nm) or ambient exposure (parent TCS and TCC), we found that the LC50s of UV and parent TCS and TCC (Fig. 5.1C) were similar to our previously reported LC50s for both TCS and TCC (Lenz et al., 2017). The LC50s of parent and UV exposed TCS were 4.27 mg/L (95% CI: 3.68, 4.89) and 3.49 mg/L (95% CI: 3.13, 3.84), respectively. The LC50s of parent and UV exposed TCC were 0.94 mg/L (95% CI: 0.74, 1.20) and 0.88 mg/L (95% CI: 0.67, 1.16), respectively. The acute toxicity of both TCS and TCC increased with UV exposure (Fig. 5.1). After 168 hours of UV exposure, the acute toxicity of TCS and TCC was 0.81 mg/L (95% CI: 0.68, 0.98) and 0.043 mg/L (95% CI: 0.034, 0.055), compared to unexposed TCS (LC50 of 3.65 mg/L) and TCC (LC50 of 0.91 mg/L). Furthermore, the parent TCC (LC50 of 0.58 mg/L) was more toxic than the unexposed TCC (LC50 of 0.91 mg/L), while the acute toxicity of parent TCS (LC50 of 3.64 mg/L) was comparable to the unexposed TCS (LC50 of 3.65 mg/L) (Fig. 5.1C). The difference in LC50 of worms exposed to UV TCS and TCC compared to parent TCS and TCC was significant ($p<0.05$).

*Impact on reproduction in the worm*

TCS and TCC exhibited significant reproductive toxicity in adult *C. elegans* at all time points (Fig. 5.2). Both UV and ambient exposure for 168 hours significantly decreased the brood size of *C. elegans*

Figure 5.2. Effect of TCS, TCC, and their photodegradation products on the number of progeny per wildtype *C. elegans*.




C

| Exposure | Brood Size (95% CI; mg/L) | % Difference | Exposure | Brood Size (95% CI; mg/L) | % Difference |
|---|---|---|---|---|---|
| Control | 200 (194, 207) | | Control | 200 (194, 207) | |
| Parent TCS 0.1 mg/L | 142 (139, 146)* | -28.9% | Parent TCC 0.01 mg/L | 137 (132, 142)* | -31.7% |
| UV TCS 0.1 mg/L | 108 (104, 111)* | -46.1% | UV TCC 0.01 mg/L | 91 (85, 96)* | -54.7% |

(A) TCS. (B) TCC. (C) Mean brood size [95% confidence intervals (CI)] and percent difference. "*" indicates significant difference from controls (p<0.05). The toxicological effect of TCS, TCC, and their photodegradation products on reproduction has been shown to be concentration-dependent (Lenz et al., 2017). UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) had a greater effect on the brood size of *C. elegans* than parent TCS (0.1 mg/L) and TCC (0.01 mg/L). The difference in brood size of worms exposed to UV TCS and TCC compared to parent TCS and TCC was significant (p<0.05).

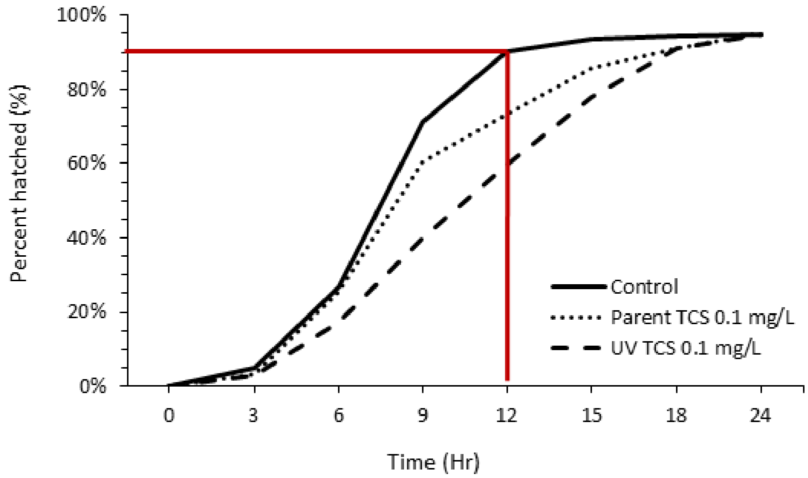
compared to controls. After 168 hours of exposure, UV TCS (0.1 mg/L) and TCC (0.01 mg/L) exhibited increased reproductive toxicity compared to parent TCS and TCC (Fig. 5.2, one-way AONVA, $p < 0.05$). Worms exposed to parent TCS and TCC had an average brood size of 142 ± 4 (-28.9%) and 137 ± 5 (-31.7%), respectively, significantly lower than the controls 200 ± 7 (Fig. 5.2C, one-way AONVA, $p < 0.001$). Worms exposed to UV TCS (0.1 mg/L) and TCC (0.01 mg/L) had an average brood size of 108 ± 4 (-46.1%) and 91 ± 6 (-54.7%), respectively, significantly lower than the controls and parent exposures (Fig. 5.2C, one-way AONVA, $p < 0.001$). UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) had a greater effect on the brood size of *C. elegans* than parent TCS (0.1 mg/L) and TCC (0.01 mg/L). The difference in brood size of worms exposed to UV TCS and TCC compared to parent TCS and TCC was significant ($p<0.05$).

*Impact on worm hatching and growth*

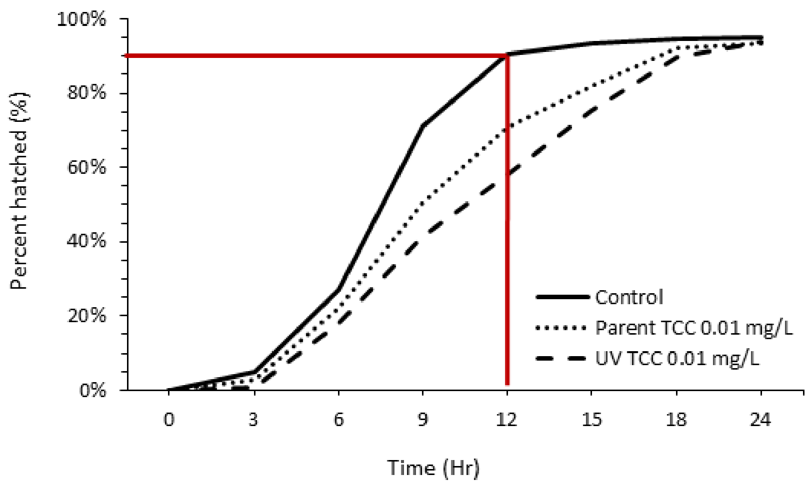
We examined the effects of TCS, TCC, and their degradation products on embryogenesis, the time from fertilization to egg hatching, via the examination of hatching time of *C. elegans* eggs (Lenz et al., 2017). In *C. elegans*, hatching time, or ex utero development, was defined as time from egg to hatching, which takes approximately 9–10 hours at 20 °C (Altun and Hall, 2018). As shown in Figure 3, at 12 hours, approximately 90% of control eggs were hatched, whereas less than 70% of eggs exposed to parent TCS (0.1 mg/L) and TCC (0.01 mg/L) and less than 60% of eggs exposed to UV TCS (0.1 mg/L) and TCC (0.01 mg/L) had hatched, indicating a significant delay in hatching. The calculated average hatching time of controls was 9.0 hours (95% CI: 8.8, 9.1). The average hatch time of parent and UV TCS was 10.4 hours (95% CI: 10.1, 10.7) and 12.0 hours (95% CI: 11.7, 12.3), respectively, while the average hatch time of parent and UV TCC was 10.8 hours (95% CI: 10.5, 11.0) and 12.1 hours (95% CI: 11.8, 12.4), respectively (Fig. 5.3C, one-way AONVA, $p < 0.05$). These findings indicate that TCS and TCC exposed to UVA (365 nm) for 168 hours had a greater impact on hatching time than parent TCS (0.1 mg/L) and TCC (0.01 mg/L). UV TCS (0.1 mg/L) and TCC (0.01 mg/L) extended the hatching time by 3 hours compared to

Figure 5.3. Effect of TCS, TCC, and their degradation products on the hatching rate of eggs from wildtype *C. elegans*.

A




B




C

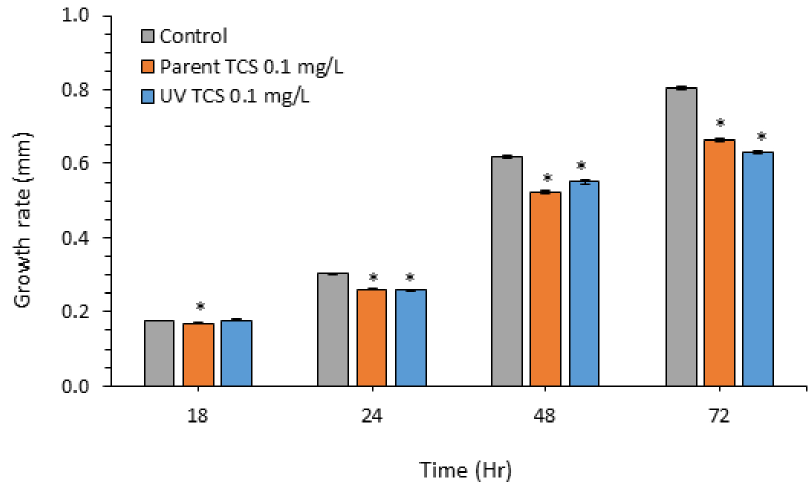
| Exposure | Average Hatch Time (95% CI; Hr) | Difference (Hr) | Exposure | Average Hatch Time (95% CI; Hr) | Difference (Hr) |
|---|---|---|---|---|---|
| Control | 9.0 (8.8, 9.1) | | Control | 9.0 (8.8, 9.1) | |
| Parent TCS 0.1 mg/L | 10.4 (10.1, 10.7)* | 1.5 | Parent TCC 0.01 mg/L | 10.8 (10.5, 11.0)* | 1.8 |
| UV TCS 0.1 mg/L | 12.0 (11.7, 12.3)* | 3.0 | UV TCC 0.01 mg/L | 12.1 (11.8, 12.4)* | 3.1 |

(A) TCS. (B) TCC. (C) Average hatching time [95% confidence intervals (CI)] and percent difference compared to controls. "*" indicates significant difference from controls (p<0.05). UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) extended the hatching time by 3 hours compared to controls. Parent TCS and TCC impacted the hatching rate of eggs by less of an extent than UV exposed TCS (0.1 mg/L) and TCC

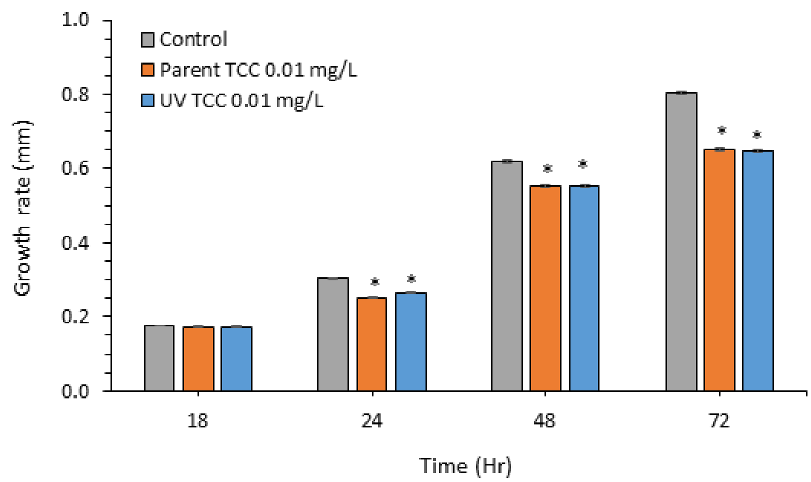
(0.01 mg/L). The difference in average hatch time of eggs exposed to UV TCS and TCC was significantly delayed compared to parent TCS and TCC ($p<0.05$).

Figure 5.4. Effect of TCS, TCC, and their degradation products on the growth rate of larval wildtype *C. elegans*.

A




B




C

| Time | Control | Parent TCS 0.1 mg/L | UV TCS 0.1 mg/L | Parent TCC 0.01 mg/L | UV TCC 0.01 mg/L |
|---|---|---|---|---|---|
| 18 hr | - | -4.0%* | 0.3% | -2.3% | -2.2% |
| 24 hr | - | -14.0%* | -14.6%* | -17.0%* | -12.6%* |
| 48 hr | - | -15.5%* | -11.0%* | -11.0%* | -10.6%* |
| 72 hr | - | -17.2%* | -21.4%* | -18.6%* | -19.5%* |

(A) TCS. (B) TCC. (C) Percent difference compared to controls. "*" indicates significant difference from controls ($p<0.05$). The parent and UV exposed compounds (TCS and TCC) decreased growth rate by >17% at 72 hours. Both UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) had a greater impact on the growth rate than parent TCS and TCC. The difference in impact was greater between parent TCS and UV exposed TCS. The difference in average growth rate of worms exposed to UV TCS was significantly decreased compared to parent TCS at 18 hr, 48 hr, and 72 hr ($p<0.05$). The difference in average growth rate of worms exposed to UV TCC was significantly decreased compared to parent TCC at 24 hr ($p<0.05$).

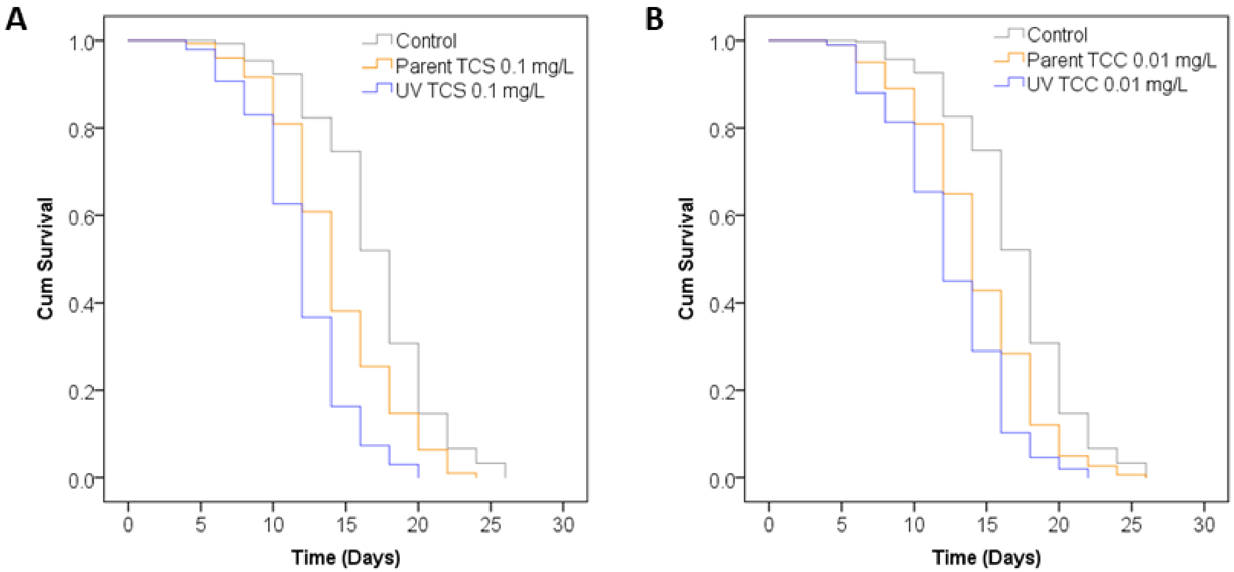
controls (Fig. 5.3, one-way AONVA, $p < 0.05$). The difference in average hatch time of eggs exposed to UV TCS and TCC was significantly delayed compared to parent TCS and TCC ($p<0.05$).

Larval worms examined for hatching time were further examined for the potential developmental toxicity of TCS, TCC, and their environmental degradation products on the developmental process of *C. elegans*. As shown in Figure 4, exposure to UV and parent TCS and TCC caused growth delay of the worms as indicated by decreased body length in exposed worms than control worms at each time point (one-way ANOVA, $p < 0.05$). For example, after 24 hours, parent and UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) decreased the body length of larval worms by 12.6% to 17% (Fig. 5.4, one-way ANOVA, $p < 0.05$). After 24 hours of exposure, parent TCC had a greater impact on larval growth rate than UV TCC, while parent and UV TCS have a similar effect on the growth rate of worms. After 72 hours, both parent and UV TCS and TCC decreased the body length of larval worms by >17% (Fig. 5.4, one-way ANOVA, $p < 0.05$). After 72 hours, both UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) had a greater impact on the body length than parent TCS and TCC. Parent and UV TCS (0.1 mg/L) decreased the growth rate of worms by 17.2% and 21.4%, respectively, compared to controls after 72 hours; while parent and UV TCC (0.01 mg/L) decreased growth rate by 18.6% and 19.5%, respectively (Fig. 5.4, one-way ANOVA, $p < 0.05$). The difference in impact was greater between parent and UV exposed TCS than with TCC. UV TCS caused the greatest decrease in the growth rate of worms (21.4%). The difference in average growth rate of worms exposed to UV TCS was significantly decreased compared to parent TCS at 18 hr, 48 hr, and 72 hr ($p<0.05$). The difference in average growth rate of worms exposed to UV TCC was significantly decreased compared to parent TCC at 24 hr ($p<0.05$).

*Impact on lifespan of the worm*

Both parent and UV exposures, 168 hour exposures, decreased the average lifespan of worms (Fig. 5.5). Control worms had an average lifespan of 17 days (95% CI: 16.6, 17.5). Worms exposed to

Figure 5.5. Effect of TCS, TCC, and their degradation products on lifespan in wildtype *C. elegans*.




C

| Exposure | Average Lifespan (Days; 95% CI) | Difference (Days) | Exposure | Average Lifespan (Days; 95% CI) | Difference (Days) |
|---|---|---|---|---|---|
| Control | 17.0 (16.6, 17.5) | | Control | 17.0 (16.6, 17.5) | |
| Parent TCS 0.1 mg/L | 14.3 (13.8, 14.7)* | -2.7 | Parent TCC 0.01 mg/L | 14.4 (13.9, 14.9)* | -2.7 |
| UV TCS 0.1 mg/L | 12.0 (11.6, 12.3)* | -5.1 | UV TCC 0.01 mg/L | 12.5 (12.0, 12.9)* | -4.5 |

(A) TCS. (B) TCC. (C) Mean lifespan [95% confidence intervals (CI)] and percent difference compared to controls. "*" indicates significant difference from controls ($p<0.05$). UV exposed TCS (0.1 mg/L) and TCC (0.01 mg/L) decreased the average lifespan by at 5.1 and 4.5 days, respectively, compared to controls. UV exposed TCS and TCC decreased lifespan by a greater extent than parent TCS (0.1 mg/L) and TCC (0.01 mg/L). The difference in average lifespan of worms exposed to UV TCS and TCC compared to parent TCS and TCC was significant ($p<0.05$).

parent TCS (0.1 mg/L) and TCC (0.01 mg/L) had a mean lifespan of 14.3 days (95% CI: 13.8, 14.7) and 14.4 days (95% CI: 13.9, 14.9), respectively, corresponding to a 2.7 day decrease in mean lifespan compared to controls. Worms exposed to UV TCS (0.1 mg/L) and TCC (0.01 mg/L) had a mean lifespan of 12.0 (11.6, 12.3) and 12.5 (12.0, 12.9), respectively. UV TCS and TCC decreased the mean lifespan by 5.1 and 4.5 days, respectively, compared to controls. Furthermore, UV exposed TCS and TCC were more decreased the mean lifespan by a greater extent than parent TCS and TCC, indicated by a 2.4 days and 1.8 days decrease between UV and parent exposures (Fig. 5.5). The difference in average lifespan of worms exposed to UV TCS and TCC compared to parent TCS and TCC was significant ($p<0.05$).

**Discussion**

TCS and TCC are known as a source of toxic and carcinogenic degradation products (Pycke et al., 2014). However, there is limited research on the environmental and public health implications of exposure to TCS, TCC, and their degradation products, despite the fact TCS and TCC are halogenated aromatic hydrocarbons with similar chemical properties as known toxic and carcinogenic compounds (i.e., polychlorinated biphenyls, dioxins, furans, hexachlorophene, polybrominated diphenyl ethers, and bisphenol A) (Halden, 2014; Dhillon et al., 2015). This lack of information on the long-term effects of exposure to TCS, TCC, and their transformation products led to the recommendations made in *The Florence Statement on TCS and TCC* of 2017, a consensus signed by over 200 experts around the globe. One of the main recommendations was the need for the evaluation of the safety of TCS and TCC transformation products from manufacturing to environmental release (Halden et al., 2017). Here we assessed the effects of TCS and TCC degradation on toxicity and found that the degradation of TCS and TCC by UV and at room temperature (without UV) increases the toxicity of TCS and TCC over 168 hours. We found that UV exposed TCS and TCC were completely degraded when exposed to UVA (365 nm) for 168 hours. Based on the literature, TCS and TCC are degraded by UV into dioxins, furans, and chloroanilines (Cawley et al., 2009; Guerard et al., 2009; Wenk et al., 2011; Wenk et al., 2012; Ding et al., 2013; Ding et al., 2014).

Aranami and Readman (2017) found that TCS was substantially photodegraded in freshwater and sea water under light conditions compared to pure water and dark conditions. The half-life of TCS in freshwater and sea water are ∼8 days and ∼4 days, respectively. Dioxins were detected in both freshwater and sea water after 3 days of irradiation (Aranami and Readman, 2007). Furthermore, TCS exposure at 254 and 365 nm transformed TCS by 90 to 98% and 78 to 90%, respectively (Son et al., 2007). Son and colleagues (2007) detected intermediate transformation products chlorophenol, dichlorophenol and phenol intermediates in all experiments, while the intermediate transformation products dibenzodichloro-p-dioxin and dibenzo-p-dioxin where only detected in experiments using 365 nm exposure (Son et al., 2007). Additionally, this study found that 75% of TCS was transformed within 20 minutes of UVA exposure, compared to 82% of TCS by a $TiO_2$ photocatalysis (Son et al., 2009). TCS was degraded by UVA exposure in the presence of the radical scavenger 2-propanol, but the degradation was significantly reduced (Son et al., 2009). This suggests that the transformation of TCS occurs via radicals (photooxidation), which further degrade the intermediates into environmentally persistent polychlorodibenzo-p-dioxins (PCDDs) (i.e., 1,2,8-trichlorodibenzo-p-dioxin, 1,2,3,8-tetrachlorodibenzo-p-dioxin, and 2,3,7-trichlorodibenzo-p-dioxin) (Son et al., 2009; Yueh and Tukey, 2016).

There is limited research on the photofate of TCC compared to TCS, which is degraded by both natural sunlight and artificial UV light (Guerard et al., 2009; Ding et al., 2013; Ding et al., 2014). The photolytic half-life of TCC exposed to UVA is approximately 24 hours (Guerard et al., 2009) and degraded into mono- and di-choroanilines, as well as chloroiso-cyanatobenzene compounds (Ding et al., 2014; Trouts and Chin, 2015; Albanese et al., 2017). The degradation of TCC occurs indirectly via side chain reactions of photosensitizers (e.g., dissolved organic matter (DOM)) with reactive photo-generated species (Trouts and Chin, 2015). The most ubitiquous photosensitizer in sunlit surface water is DOM, generating reactive oxygen species (ROS) and non-ROS transients when irradiated (Trouts and Chin, 2015; Zhang et al., 2016). A 2017 study found that chlorinated anilines and isocyanates were produced by the indirect photolysis of TCC in the presence of DOM (Albanese et al., 2017). Furthermore, the toxicity of degradation products formed by direct photolysis and indirect photolysis of TCC in the presence of DOM had LC50 values of 2.67 ± 0.6 μM and 0.032 ± 0.015 μM, respectively in Daphnia magna. The LC50 of parent TCC in D. magna was 0.087 ± 0.3 μM. The degradation products formed by the indirect photolysis of TCC was significantly more toxic than parent TCC and the degradation products formed by direct photolysis (Trouts and Chin, 2015).

The nematode *C. elegans* is an ecologically relevant model to examine the effects of chemical exposures via ingestion, of both food and water, that pose a potential hazard to the health of organism through the entire life cycle, embryogenesis to death (Page and Johnstone, 2007, O'Reilly et al., 2014). Studies in this worm model examine exposure via ingestion because of the presence of a cuticle that makes absorption of toxins difficult (Hunt, 2017). We found that increased durations of UVA exposure, approximately 95% of UV that reaches the Earth (WHO, 2019), were associated with increased acute lethality (Fig. 5.1). The increased acute toxicity of UV TCS and TCC was associated with increased toxicity in reproduction, development, and lifespan. In *C. elegans*, TCS and TCC exhibit toxicity in a concentration-dependent manner. UV exposed TCS and TCC decreased brood size by 46% and 55%, respectively, compared to controls (Fig. 5.2). These findings are comparable to the effect of the highest concentration of unexposed TCC (0.5 mg/L) was reported in our previous study, a 50% decrease in brood size compared to controls (Lenz et al., 2017). This decrease in number of progeny can cause downstream effects on embryogenesis and growth (Gardner et al., 2013).

Embryogenesis in nematodes is the time from fertilization to egg hatching (Gilbert and Sunderland, 2000). In *C. elegans*, embryogenesis takes 12-13 hours at 20 °C and consists of both utero

(2–3 hours) and ex utero (9–10 hours) development (Harlow et al., 2016). Our knowledge of embryogenesis in *C. elegans* provides a convenient measure, hatching time, to study the potential impact of chemicals on the worm's developmental process, such as cell fate specification, tissue formation, and morphogenesis (Corsi, 2006; Harlow et al., 2016). We found that UV TCS (0.1 mg/L) and TCC (0.01 mg/L) delayed the hatching time by 3 hours compared to controls, while parent TCS and TCC delayed the hatch time by 1.5 and 1.8 hours, respectively (Figure 5.3C). Our previous study found that TCS (0.1 mg/L) and TCC (0.01 mg/L) delayed the hatch time by 2.0 and 1.9, respectively (Lenz et al., 2017), which are similar to the delay in hatch time of parent TCS and TCC (Figure 5.3). UV exposure, for 168 hours, caused a greater effect on embryogenesis than unexposed or parent TCS and TCC. UV TCS and TCC exposure resulted in a 1 hour delay in hatch time (Figure 5.3) compared to the unexposed TCS and TCC (Lenz et al., 2017). We found that TCS, TCC, and their degradation products significantly delay in hatching time of worms, suggesting the developmental toxicity of these compounds and degradation products. Currently, the long-term effects of delayed hatching on the worm's overall health and life course are unknown. Additional research is needed to understand the mechanism of toxicity resulting delayed hatching. Another study found that imidazole, pyridazine, pyridopyrazine, and triazolopyrimidine fungicides exhibit developmental toxicity on *C. elegans* eggs (Harlow et al., 2016). These fungicides are known to disrupt microtubule dyanmics (Rathinasamy & Panda, 2006; Lamberth et al., 2012; Lamberth et al., 2013). Additional research is needed to determine the mechanism of toxicity of TCS, TCC, and their degradation products that results in delayed egg hatching.

Following the delayed hatching, we also found that TCS, TCC, and their degradation products reduced the worm's growth rate, shown by a reduction in body length compared to controls. After hatching, larval worms progress through four distinct larval stages (L1-L4) and reach adulthood at approximately 3 days (72 h) of age (Uppaluri and Brangwynne, 2015). We observed decreased body length of the worms exposed to TCS, TCC, and their degradation products at 18, 24, 48, and 72 hours

(Fig. 5.4). Stage-specific development of *C. elegans* is regulated by at least three different genetic pathways: (1) a spectrin pathway involving the *unc-70*, *sma-1*, and *spc-1*; (2) a calcineurin pathway involving *tax-6* and *cnb-1*; and (3) a transforming growth factor-b (TGF-B) pathway involving *sma-2*, *sma-3*, *sma-4*, *lon-1*, *kin-29*, and *dbl-1* (Morck and Pilon, 2006). The interference of these pathways can result in "heterochronic" growth abnormalities (Zaidel-Bar et al., 2010, Monslave et al., 2011). Additionally, an inadequate food source can halt development. We believe this to be ulikely because our worms were provided an optimal amount of food and no feeding behaviors associated with starvation were observed (Dalliere et al., 2017). Additional research is needed to determine the mechanism(s) of toxicity leading to the decrease in body size, as well as the long-term effects of the decrease in body size.

In addition to the observed developmental and reproductive toxicity, TCS, TCC, and their degradation products significantly decreased the average lifespan of *C. elegans* (Fig. 5.5). In this study, we found that TCS and TCC exposed to UVA for 168 hours decreased the average lifespan 5.1 and 4.5 days, respectively, compared to controls (Fig. 5.5). UV exposed TCS and TCC decreased lifespan by a greater extent (5.1 and 4.5 days, respectively) than parent TCS (0.1 mg/L) and TCC (0.01 mg/L), by 2.7 days (Fig. 5.5). The increased toxicity on *C. elegans* lifespan indicates the presence of degradation products in the parent TCS and TCC solutions. Additional research is needed to determine the specific degradation products of all exposure groups. Additionally, aging and lifespan are processes regulated by complex interactions of genetics, environment, and other nongenetic factors (Uno and Nishida, 2016). Exposure to TCS, TCC, and their degradation products significantly reduced the growth, reproduction, and lifespan of worms, suggesting the potential involvement of insulin/insulin-like growth factor (IGF) signaling.

IGF is an important regulatory pathway that plays a vital role in prenatal growth, fertility, regulating fat storage and blood glucose, stress response, and aging in the worm (Laviola et al., 2008, Zhang and Liu, 2014; Gao et al., 2017). IGF signaling is controlled by the activation of the IGF receptor, *daf-2*. Once *daf-2* is activated by the binding of a substrate, the signaling cascade involving *age-1* (phosphoinositide 3-kinase), *pdk-1* (phosphoinositide-dependent protein kinase-1) and *akt-1/2* (protein kinase B) is initiated resulting in the phosphorylation of *daf-16* (Murphy et al., 2003, Murphy, 2013). The phosphorylation of *daf-16* inhibits this transcription factor's relocation into the nucleus (Ogg et al., 1997). The overactivation of IGF signaling is associated with accelerated aging and increased mortality in the worm (Fadini et al., 2010). Our next step is to examine the impact of the degradation products of TCS and TCC on IGF signaling and compare the results to their parent compounds, discussed in Chapter 4.

In a previous study, we reported the finding of the development of a severe vulval integrity defect (Avid) in adult worms during the aging process when exposed to a type of cyanopeptides, known as anabaenopeptins (Lenz et al., 2018). A study by Leiser (2016) described this Avid phenotype and hypothesized that the germline of *C. elegans* is an important modulator of Avid due to the association of increased Avid formation and a loss of oocyte development (Leiser et al., 2016). In our study, we did not observe a loss of oocytes following exposure to TCS, TCC, and their degradation products; however, we did not observe the presence of an Avid phenotype. This suggests that the development of Avid phenotypes may (1) depend upon certain exposures, (2) be influenced by changes in function of the 16 egg-laying muscles and hermaphrodite-specific neurons (e.g., motorneurons and the ventral nerve cord), and (3) require a combination of biological factors (i.e., fat accumulation, fat metabolism, systemic iron overload, and ferritin levels) for development of Avids (Seidell et al., 1990; Collins and Koelle, 2013; Hyun et al., 2016, Wang et al., 2016). Future studies will examine the mechanism(s) of Avid development and the link between specific environmental contamininants and the age-related vulval defect.

## Conclusion

The UV exposure that occurs in the environment increases the toxicity of TCS and TCC, which was observed through a decrease in LC50, reproduction, and lifespan. These findings suggest that TCS and TCC may pose significant health risks to aquatic organisms. Our next steps are to characterize the degradation products that were formed during the UV exposure and understand the underlying mechanism(s) of observed reproductive toxic effects of TCS, TCC, and their degradation products.

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# CHAPTER 6:

## Discussion/Conclusion

Environmental toxicology, like other branches of science, is an ever-changing field of research that is essential for the assessment of new chemicals that are continually introduced to industrial production and human life (Connon et al., 2012; Sturla et al., 2014). The assessment of these chemicals for potential toxicity is crucial for the determination of safe levels of exposure to humans and presents an urgent and ongoing issue (Gou et al., 2015). The scientific community is under pressure to re-evaluate the use of specific animal models and methods for measuring toxicity (Burden et al., 2015; Gao et al., 2018). In recent years, several studies have focused their research on antimicrobials and cyanopeptides. Here we investigated the toxicological effects of two types of commonly detected environmental contaminants, antimicrobials (triclosan and triclocarban) and cyanopeptides (anabaenopeptins and cyanopeptolins), using the model organism *Caenorhabditis elegans* (*C. elegans*). In our current analysis, we have shown that exposure to environmentally-relevant levels of cyanopeptides and antimicrobials affects the health of the worm throughout its life, in utero to death.

**Cyanopeptides**

Cyanobacterial blooms pose a potential threat to environmental and public health, as well as local economies, due to the production of highly toxic secondary metabolites (cyanopeptides) (Pick, 2016; Wilkinson et al., 2018; Kimambo et al., 2019). These cyanopeptides, or cyanotoxins, have been detected in drinking water and food sources at concentrations reaching mg/L, posing a significant public health risk (Gkelis et al., 2015; Kurmayer et al., 2016). There are more than 600 identified cyanopeptides, such as anabaenopeptins, cyanopeptolins, and microcystins (MCs) (Gkelis et al., 2015). Some of these cyanopeptides are produced with MCs, most commonly known cyanopeptide, and in some cases are detected at equal or higher concentrations than MCs (Beversdorf et al., 2017, 2018; Janssen, 2019; Larsen et al., 2019). While the World Health Organization has set guideline values detectable MC in drinking and recreational water, there is little known about the potential risk many of these peptides may pose to public and environment (Welker and Von Dohren, 2006; WHO, 2008).

Growing on research that suggests that cyanopeptides pose a potential threat to environmental and public health, our research found that anabaenopeptins (AP-A, AP-B, and AP-F) and cyanopeptolins (CYP-1007, CYP-1020, and CYP-1041) induced significant toxicity effects to the worm at concentrations 100 times lower than those found in the environment. The observed toxic effects include reduced reproduction, delayed hatching, decreased growth rate, shortened lifespan, as well as severe aging-related vulval integrity detects. At an environmentally relevant concentration (10 μg/L) APs were the most toxic, followed by CYPs, which showed comparable toxicity to MC-RR. Microginin 690 was the least toxic. These findings suggest that APs and CYPs may pose a significant health risk to aquatic organisms. Additional toxicological studies are needed to more completely understand the potential impact of these cyanopeptides on the environment and human health. Future studies should aim to understand whether these observed toxicity in the worm can be related to the known protease inhibition properties of these cyanopeptides, as well as whether these toxic effects are observed in other species. Furthermore, given the frequent co-occurrence of these APs and CYPs with MCs as well as their comparable or even greater toxicity than MCs, potential recreational and drinking water guidelines need to account for these cyanopeptides.

**Antimicrobials**

Antimicrobials, such as triclosan (TCS) and triclocarban (TCC), are used in numerous personal care products, consumer products, and medical devices (i.e., cosmetics, food packaging, plastics, children's toys, clothing, building materials, textiles, and medical devices) (Halden, 2014). TCS and TCC are known endocrine disruptors that are ubiquitously found in the environment and have been detected in tissues and fluids of many organisms (Halden and Paull, 2005; Dann and Hontela, 2011; Dhillon et al., 2015). Exposure to TCS and TCC is associated with reproductive and developmental toxicity, increased obesity risk, infertility, and development and proliferation of cancer cells (Vandenberg et al., 2012; Higgins et al., 2011; Haggard et al., 2016; US EPA, 2017). Due to the growing concern regarding the use of antimicrobials by the general public, the FDA banned 19 chemicals chemicals (e.g., TCS, TCC, other halogenated aromatics, and methylbenzethonium chloride) from antibacterial soaps in the fall of 2016 (FDA, 2016). Following this ruling by the FDA, a group of more than 200 scientists and medical professionals released a statement, known as *The Florence Statement on TCS and TCC* (Halden et al., 2017). This statement called for further restrictions on the use of antimicrobials, as well as addressed gaps in our knowledge regarding the adverse effects of exposure to TCS and TCC (Halden et al., 2017).

In 2017, *The Florence Statement on TCS and TCC* was published and summarized decades of research and raised concerns regarding the long-term health and ecological impacts of antimicrobials and the need for greater transparency during product development and toxicity testing before a chemical is used in a commercial product (Halden et al., 2017). In addition, the statement highlighted 10 important points and gaps in our knowledge of TCS and TCC and presented 4 recommendations. The research shared here addressed the following recommendations and gaps in our knowledge of these two chemicals presented by *The Florence Statement on TCS and TCC*: (1) examine the reproductive and developmental impacts of TCS and TCC; (2) evaluate the safety of TCS, TCC, and their transformation products throughout the entire product life cycle, including environmental release; and (3) call the international community to limit the production and use of TCS and TCC (Halden et al., 2017).

*Reproductive and developmental toxicity*

TCS and TCC are known endocrine disruptors that were found to cause developmental and reproductive toxicity, decreased longevity, lipid accumulation, and altered activity of genes involved in the worm's stress response (Lenz et al., 2017; Garcia-Espineira et al., 2018). In addition, TCS- and TCC-induced toxicity was further examined via genetic analysis with a series of worm mutants and gene expression in wildtype worms. This study found that the loss or reduction of activity of IGF genes in mutant strains, especially *age-1, pdk-1,* and *akt-1,* were associated with decreased toxicity of TCS and TCC. Furthermore, TCS and TCC increased the expression of genes involved with IGF signaling and vitellogenesis. TCC increased the expression of *daf-2*, the IGF receptor, as well as other IGF genes. However, TCS does not increase the activity of *daf-2*. It is not clear how TCS increases the activity of IGF signaling genes downstream of *daf-2*. It is possible that TCS and TCC could bypass the IGF receptor due to their lipophilic properties, while TCC also increases the expression of the IGF receptor. Additional research is needed to determine if the lipophilic properties of TCS and TCC allow these chemicals to bypass the IGF receptor, *daf-2*.

In addition, TCS and TCC increased the expression of *vit-2*, a vitellogenesis gene. The expression of *vit-2* has been shown to inhibit yolk protein production resulting in increased intestinal lipid content (Ezcurra et al., 2018). Yolk synthesis, lipid redistribution, and intestinal atrophy are *daf-2* and *daf-16* dependent processes (Ezcurra et al., 2018). An age-related vulval defect, avid phenotype, is hypothesized to be the result of reproduction causing the vulva to be a site of weakness that is exacerbated by the failure to shut down the production of yolk, protein, and fat (Leiser et al., 2016). Avid phenotypes are associated with the loss of oocyte production and decreased lifespan in *C. elegans* (Leiser et al., 2016). In our study, we found that mutants with decreased longevity in controls were associated with increased frequency of an avid phenotype and decreased reproduction. Worms with a mutation resulting in the loss of function of IGF signaling genes (e.g., *daf-2, age-1, pdk-1, akt-1/2,* and *daf-16*) did not develop this avid phenotype. Avids were not observed in wildtype worms exposed to TCS and TCC; however exposure to cyanopeptides did yield this development (Ch. 2). Additionally, we found that the expression of *vit-2* was increased by both TCS and TCC in wildtype worms (Fig. 4.7), but we did not examine the expression of *vit* genes in the mutants with increased frequency of avid phenotype.

To the best of our knowledge, this is the first study to examine the effects of TCS and TCC on IGF signalling. Additional research is needed to determine the role of IGF signalling and the expression of *vit* genes in the production of avid phenotypes. Future research should examine the expression of *vit* genes in *ist-1(ok2706), daf-18(ok480), daf-18(e1375),* and *akt-1(mg144)* control worms, as well as worms exposed to environmental contaminants known to induce this avid phenotype. Moreover, it is hypothesized that this avid phenotype may be analogous to obesity in higher organisms (Leiser et al., 2016), which is an adverse outcome associated with the overactivation of IGF, decreased lifespan, and decreased reproduction (Seidell et al., 1990; Berryman et al., 2013; Lewitt et al., 2014). Additional research is needed to determine if the avid phenotype is analogous to obesity. Future studies are needed to examine the molecular effects of TCS and TCC exposure at different ages, elucidate the potential mechanism(s) of toxicity resulting in Avid phenotype, and determine the transgenerational effects (See Appendix A and B for preliminary data). Additionally, future studies should examine the effect of TCS and TCC toxicity based on the exposure window, such as examining the development of Avids and gene expression using an exposure window beginning at egg stage.

Given TCS and TCC increase IGF signaling, it is important to consider more localized effects TCS and TCC toxicity may have. It has been well established that IGF receptors in the brain (Lewitt & Boyd, 2019). IGF signaling is an essential factor for early brain development and healthspan later in life, such as reproduction, mental health, and lipid synthesis and storage (Wrigley et al., 2017). During embryonic development, IGF expression occurs in the cortex, subventricular zone-olfactory bulb, hippocampus, cerebellum, hypothalamus, and spinal cord (O'Kusky & Ye, 2012; Nieto-Estevez et al., 2016). Once born and throughout adulthood, IGF expression occurs in the subventricular zone-olfactory bulb, hippocampus, cerebellum, and the choroid plexus (Falcao et al., 2012; Zeigler et al., 2015). In addition, IGF can act in the brain in an endocrine, paracrine, or autocrine manner (Nieto-Estevez et al., 2016). This is to the ability of IGF to (1) pass from the blood to the cerebrospinal fluid through the lipoprotein receptor-related protein 2 (LRP2); (2) cross the blood-brain-barrier by binding to the IGF receptor present in endothelial cells; and (3) be picked up in the brain either by astrocytes to be transferred to neurons or directly by neurons (Nieto-Estevez et al., 2016; Grey &Thorner, 2017). Additional research is needed to determine how TCS and TCC exposure impacts IGF activity in the brain.

*TCS, TCC, and their degradation products*

TCS and TCC enter the environment everyday where these chemicals are exposed to solar ultraviolet (UV) radiation that can degrade these compounds and result in the formation of toxic and carcinogenic products, such as dioxins, furans, chlorophenols, and anilines (Latch et al., 2003; Aranami and Readman, 2007; Buth et al., 2009; Buth et al., 2010; Pycke et al., 2014; Alvarez-Rivera et al., 2016; Lehutso et al., 2017). These compounds have been widely detected in the environment, including surface water, soil, and sediment (Allmyr et al. 2008; Halden et al., 2014). The examination of the environmental risks TCS, TCC, and their degradation products should not be neglected (Lehutso et al., 2017). Thus, we examined the effects UV exposure has on the toxicity of TCS and TCC using our established organismal and molecular endpoints, such as lethality, reproduction, hatching time, growth rate, and lifespan. Our findings showed that the TCS and TCC exposed to UV exhibited increased toxicity. The 24 hour lethality of 168 hours of UV exposure to TCS and TCC decreased from 3.65 mg/L to 0.81 mg/L and 0.91 mg/L to 0.04 mg/L, respectively. At sublethal concentrations, TCS and TCC exposed to UV increased the sublethal toxicity in *C. elegans*. This was observed via delayed hatching and decreased reproduction and lifespan compared to ambient TCS and TCC exposure. These findings suggest that TCS, TCC, and their degradation may pose significant health risks to environmental and public health.

Our next steps are to characterize the degradation products that were formed during the UV exposure and understand the underlying mechanism(s) of observed reproductive toxic effects of TCS, TCC, and their degradation products. Preliminary data on the degradation of TCS by UV exposure shows that TCS was degraded by UV exposure compared to control (Apendix C). Additional research is needed to verify the degradation of both TCS and TCC, as well as characterize their degradation products. This knowledge will provide insights into the production of specific byproducts and the toxicity of specific mixtures of these toxic degradation products. Additionally, the established methods of characterization by mass spectrometry can be applied to future studies of environmentally persistent pollutants and their degradation products to determine the presence of specific molecules in various matrices (e.g., such as water, soil, urine, and serum), as well as the quantification and analysis of the total chemical exposure in toxicological studies. Quantifying the total chemical exposure in a model organism will provide a better understanding of the toxicological effects of the chemical of interest. This information will provide additional knowledge regarding the public health implications of exposure to these environmentally persistent pollutants and aid in the development of regulations and policies regarding the use, disposal, and cleanup of these pollutants.

*Public Health Policy*

Results from environmental toxicology studies, such as this one, can be used by policymakers as scientific evidence in support of the creation or amendment of public health policies (Guo et al., 2015). In Europe, the European Food Safety Administration (EFSA) and the European Chemicals Agency (ECHA) published their new 5 level guidance system for the identification of endocrine disrupting chemicals, which included the use of invertebrate models, such as nematodes, for the flagging of toxic chemicals and for providing scientific evidence in the creation of public health policies (Andersson et al., 2018).

The published research presented in Chapter 3 is currently being used to aid in the amendment of Environmental Conservation Law (Add §37-0115) in New York State (S1184), co-sponsored by Senators Patty Ritchie and James Sanders Jr (Ritchie & Sanders, 2019). This bill calls for the banning of certain chemicals, such as TCS, from being manufactured, used, or stored in the state of New York (Ritchie & Sanders, 2019). This bill would call for a complete ban of TCS, in New York, which would be much more expansive than the FDA ban of 2016 (FDA, 2016; Ritchie & Sanders, 2019). Additional work is needed to regulate antimicrobials, such as TCS and TCC. However, the FDA ban of use in soaps and the work being done in New York State are examples of the changing times. Furthermore, the inclusion of toxicological research using the model organism *C. elegans* by New York State, ECHA, and EFSA signifies the changing attitudes regarding invertebrate research and its role in the field of toxicology.

**Alternative Model**

Traditionally toxicology studies have employed vertebrate models, such as rodents and fish, to assess the potential toxicity of chemicals (Gao et al., 2018). This strategy has been important in establishing the Globally Harmonized System (GHS) of Classification and Labeling of Chemicals (UNECE, 2019). However, the use of vertebrate models is time-consuming, expensive, and raises ethical concerns. In recent years, regulatory agencies across the globe have pushed for the exploration of alternative animal models for toxicity testing (Krewski et al., 2010). For example, regulatory agencies in the United States are developing a list of approved alternative toxicological testing methods and a framework known as the Predictive Toxicology Roadmap for the scientific validation integration of these alternative toxicology methods (Schechtman, 2002; FDA, 2019; NTP, 2019a). The EFSA and ECHA have incorporated the use of invertebrate models for the identification of endocrine disrupting chemicals (Andersson et al., 2018). The inclusion of animal models that are less expensive and time-consuming are necessary for the screening of the ever-growing list of chemicals and for the advancement of the field of toxicology (Boyd et al., 2016).

Alternative models that are being explored for toxicological studies include nematodes (Hoss and Weltie, 2007). Nematodes have been used for decades in research; however, it wasn't until recently that these invertebrates were employed for toxicological research. Nematodes, such as *C. elegans*, can easily be used to screening chemicals, such as antimicrobials, cyanopeptides, and pharmaceuticals (Boyd et al., 2016). The available methods, well-understood biology, and extensive genetic information, short lifespan, and transparent bodies make *C. elegans* an excellent model to screen potential toxicants (Allard et al., 2013; Hägerbäumer et al., 2015). *C. elegans*-based assays can provide information on the toxicological effects of chemicals on development, the nervous and digestive systems, metabolics, tissue-to-tissue signaling, reproduction, and aging (Planchart et al., 2016; NTP, 2019a; NTP, 2019b). Many pathways involved in organismal, endocrine function, and neural development are highly conserved in invertebrates and mammals (Leung et al., 2008). However, *C. elegans* lack many mammalian organs (i.e., eyes, lungs, heart, kidney, and liver) and adaptive immunity (Hunt, 2017). This means that *C. elegans*-based assays are an excellent model for studying ingestion, or oral toxicity, but not absorption studies, due to the presence of a cuticle (Hunt, 2017). Thus, *C.elegans*-based assays need to be paired with vertebrate models to determine the full extent of a chemical's toxicological effects on an organism. However, *C. elegans*-based assays provide an excellent complement to *in vitro* and *in vivo* models that can red flag chemicals that need further examination for toxicological effects (Leung et al., 2008; Xiong et al., 2017).

The employment *C. elegans*-based assays would allow for faster screening of chemicals and allow for the appropriate stakeholders to address concerns earlier in the risk assessment process (Xiong et al., 2017). This will hopefully minimize or eliminate the use of high risk chemicals and limit their introduction into the environment (Krewski et al., 2010). A way in which early toxicity testing can be performed early in the production of chemicals and products is through the use of a high-throughput animal model, such as an invertebrate model (Szymanski et al., 2012). The use of *C. elegans*, or another invertebrate model, can provide a quick screen for new chemicals moving forward. Faster screening platforms will allow researchers, regulatory agencies, and companies the ability to flag chemicals that pose a potential risk to environmental and human health earlier in the process (Allard et al., 2013; Boyd et al., 2016; Schmeisser et al., 2017). This will allow concerns to be addressed before toxic and environmentally persist enter our environment.

The use of *C. elegans* as a model organism can be taken a step further via the use of toxicity data from *C. elegans* studies to generate a computational model that can predict chemical toxicity. There are numerous toxicology studies using *C. elegans* as a model organism and a variety of established endpoints to create a computational model (Sarma et al., 2018). For example, researchers are creating a computational model, such as OpenWorm, a model constructed using a variety of biological data to simulate biological functions of *C. elegans* in a 3D environment (Sarma et al., 2018; OpenWorm Foundation, 2018). The goal of computational models, such as OpenWorm, is to provide researchers with a platform that allows for the integration of rigorously tested simulations with existing theoretical or experimental methods (Gleeson et al., 2018; OpenWorm Foundation, 2018; Sarma et al., 2018). These methods could be applied to other organisms and assist in our understanding of biology (Sarma et al., 2018).

The future use of a computional model, such as OpenWorm, requires the use of data regarding known toxicants, such as antimicrobials and cyanopeptides. Studies like the one presented here would provid the much needed data to begin constructing a computational model for the use for studying toxicology and risk assessment. Additional work is needed to obtain extensive data on the effects of a variety known toxicants in *C. elegans* to create an accurate computational model. Future studies can use the established methods and toxicological endpoints from this study to collect the necessary data.

**Conclusion**

The research presented here addresses gaps in our knowledge of the toxic effects of both cyanopeptides and antimicrobials. This study has established several organismal and molecular endpoints that can be employed for the investigation of the toxicological effects of chemicals. *C. elegans* can provide a bridge between *in vitro* assays and mammalian toxicity testing that can speed up the chemical screening process. These results will establish a new mechanism of TCS- and TCC-induced toxicity and provide valuable information to guide future studies using vertebrate models. This information will provide additional knowledge regarding the public health implications of exposure to these environmentally pollutants and aid in the development of regulations and policies regarding the use, disposal, and cleanup of these pollutants.

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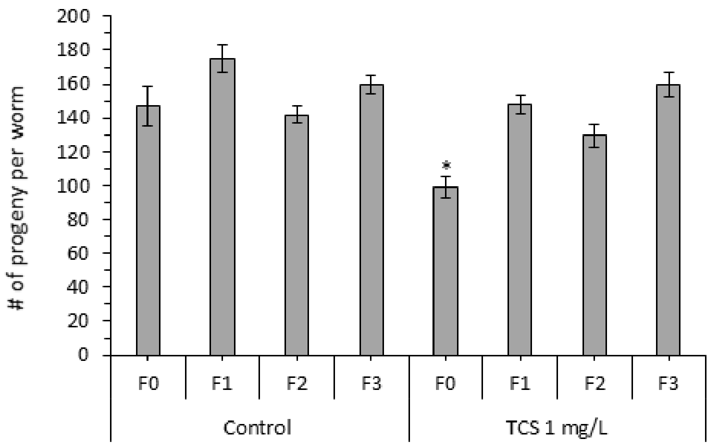
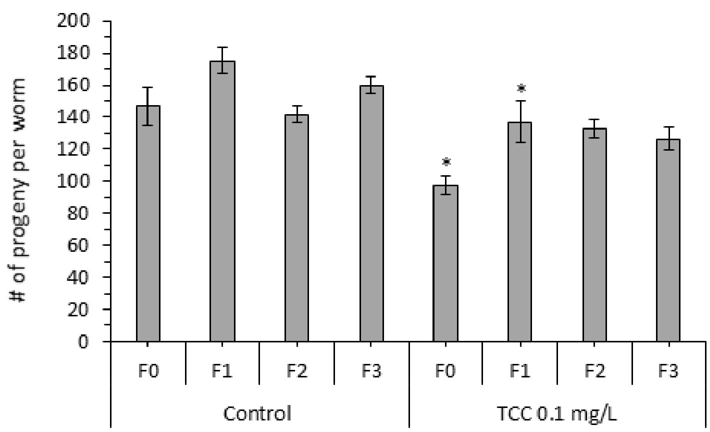
APPENDIX

Appendix A: The transgenerational impact of TCS or TCC exposure on *C. elegans* reproduction, F0 exposure.

A



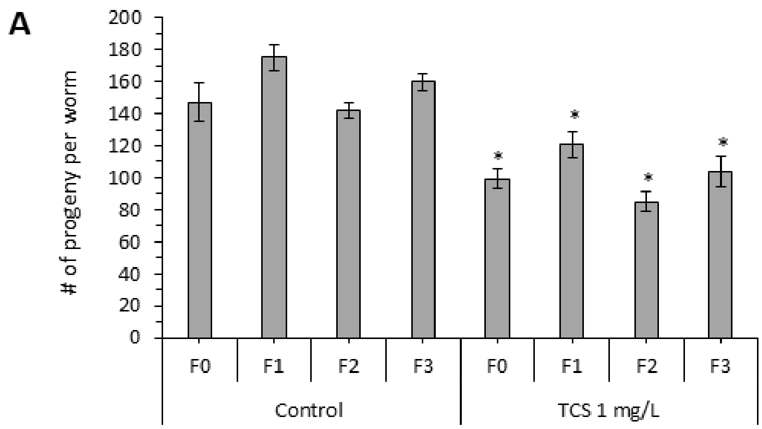

B

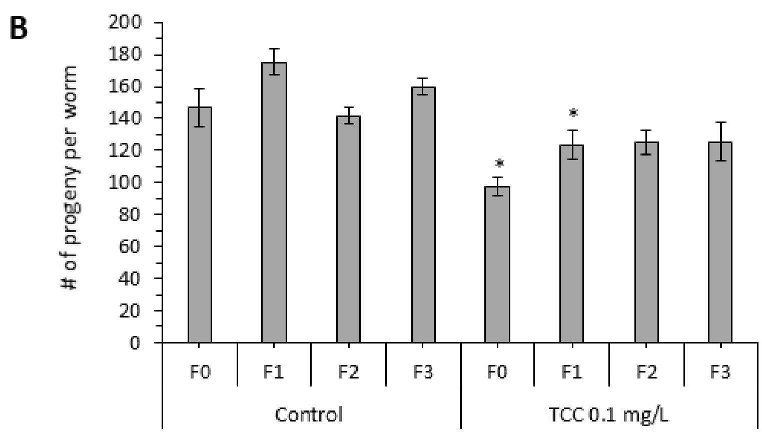



| | Generation | | | |
|---|---|---|---|---|
| | F0 | F1 | F2 | F3 |
| Control | 147<br>(116.5, 177.5) | 175.2<br>(154, 196.4) | 141.8<br>(129.2, 154.5) | 159.8<br>(145.6, 174.0) |
| TCS 1 mg/L | 99.3<br>(85.5, 113.1)* | 148<br>(134.2, 161.8) | 129.5<br>(111.4, 147.7) | 159.8<br>(141.9, 177.7) |
| TCC 0.1 mg/L | 97.3<br>(97.0, 119.0)* | 136.8<br>(104.0, 169.7)* | 132.8<br>(118.2, 147.5) | 126.5<br>(108.7, 144.3) |

(A) TCS, (B) TCC. Error bars indicate standard errors of the mean based on two independent replicate experiments (n = 2). “*” indicates significant differences according to Tukey's HSD test in conjunction with ANOVA analysis ($p < 0.05$). Plate preparations and reproduction tests were proformed using methods described by Lenz (2017). F1 generation plates were set up using the progeny of F0 from day 2 plates, after the number of progeny was recorded (6 worms per exposure with 1 worm per plate). Exposure to TCS (1 mg/L) and TCC (0.1 mg/L) occurred for only the F0 generation. Both F0 and F1 generations were followed for all of the reproductive cycle and all progeny were recorded ever other day. Following the reproductive cycle, the average number of progeny per worm was calculated by adding the number of progeny from all plates of an exposure concentration. The same procedures were followed for the F2 and F3 generations.

Appendix B: The transgenerational impact of TCS or TCC exposure on *C. elegans* reproduction, continually exposure from F0 to F3.

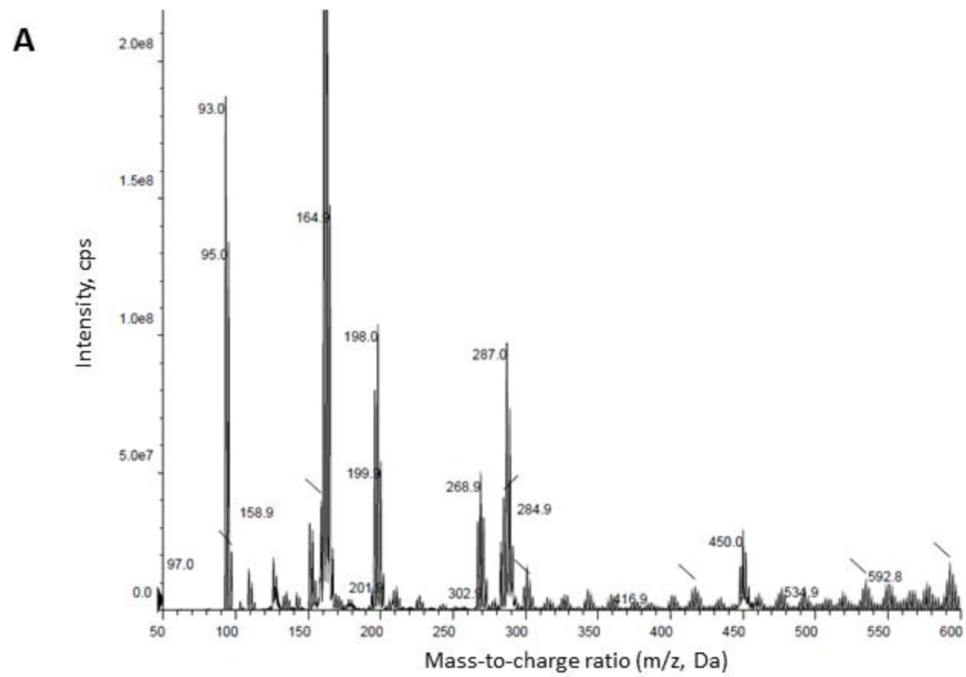


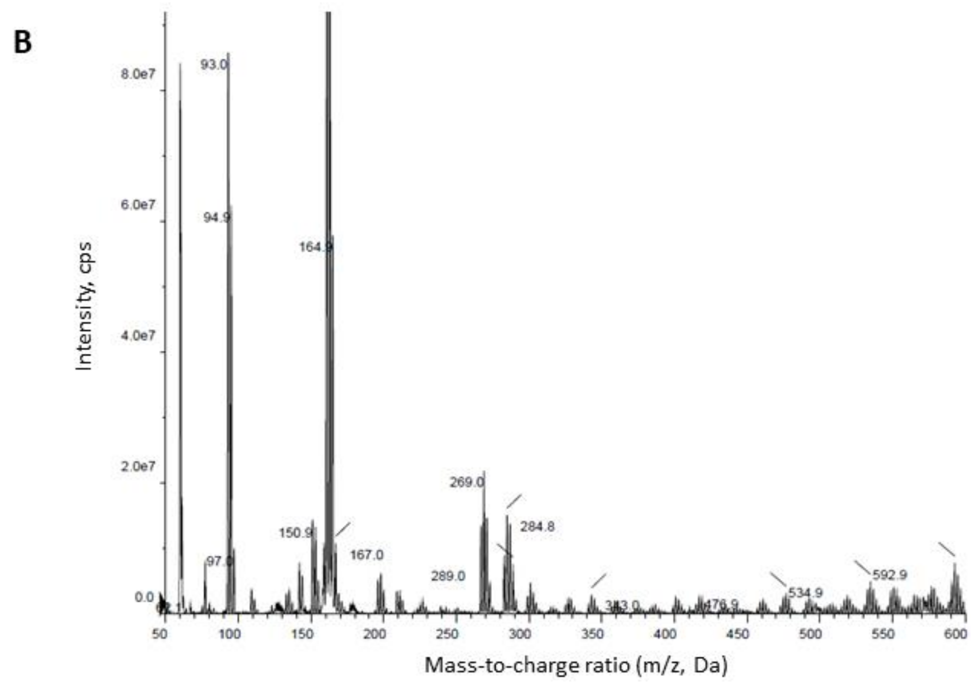




| | Generation | | | |
|---|---|---|---|---|
| | F0 | F1 | F2 | F3 |
| Control | 147 (116.5, 177.5) | 175.2 (154, 196.4) | 141.8 (129.2, 154.5) | 159.8 (145.6, 174.0) |
| TCS 1 mg/L | 99.3 (85.5, 113.1)* | 120.7 (99.6, 141.7)* | 84.83 (68.6, 101.1)* | 103.8 (79.7, 128.0)* |
| TCC 0.1 mg/L | 97.3 (97.0, 119.0)* | 123.5 (100.2, 146.8)* | 125.2 (105.2, 145.1) | 125.5 (95.2, 155.7) |

(A) TCS, (B) TCC. Error bars indicate standard errors of the mean based on two independent replicate experiments (n = 2). "*" indicates significant differences according to Tukey's HSD test in conjunction with ANOVA analysis ($p < 0.05$). Plate preparations and reproduction tests were proformed using methods described by Lenz (2017). F1 generation plates were set up using the progeny of F0 from day 2 plates, after the number of progeny was recorded (6 worms per exposure with 1 worm per plate). Exposure to TCS (1 mg/L) and TCC (0.1 mg/L) occurred for all 4 generations (F0, F1, F2, and F3). Both F0 and F1 generations were followed for all of the reproductive cycle and all progeny were recorded ever other day. Following the reproductive cycle, the average number of progeny per worm was calculated by adding the number of progeny from all plates of an exposure concentration. The same procedures were followed for the F2 and F3 generations.

Appendix C: Detection of the TCS in UV exposed samples using LC-MS/MS.




(A) TCS Control (B) UV TCS; exposed for 168 hr. TCS was detected in the TCS control, at a mass-to-charge ratio of 287, but was not detected in the 168 hr UV exposed TCS sample. Further testing needed to detect the degradation of TCS and TCC from all samples. TCS is detected at 287-288 and TCC is detected at 308.

CURRICULUM VITAE

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**PROFESSIONAL EXPERIENCE:**

| | |
|---|---|
| 2019-Present | Public Health Manager, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2019-Present | Information Center Coordinator, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2015-2019 | Research Assistant, Department of Environmental Health Sciences, Joseph J. Zilber School of Public Health, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2014-2015 | Research Assistant, Wisconsin Tobacco Prevention and Control Program, Division of Public Health, Wisconsin Department of Health and Family Services, Madison, WI. |
| 2014 | Laboratory Intern, City of Milwaukee Health Department Laboratory, Milwaukee, WI. |
| 2013-2014 | Health and Safety Policy Supervisor, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2010-2013 | Policy, Nutrition, and Marketing Intern, St. Norbert College, De Pere, WI. |

**EDUCATION:**

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| 2015-2020 | Joseph J. Zilber School of Public Health, University of Wisconsin-Milwaukee, Milwaukee, WI. *Doctorate of Philosophy Student, Environmental Health Sciences, Focus Environmental Toxicology and Risk Assessment*. |
| 2013-2015 | Joseph J. Zilber School of Public Health, University of Wisconsin-Milwaukee, Milwaukee, WI. *Master of Public Health, Environmental Health Sciences.* |
| 2009-2013 | St. Norbert College, De Pere, WI. *Bachelor of Science, Biology, Concentration in Biomedical Sciences.* |

**PUBLICATIONS:**

Ma H, **Lenz KA**, Gao X, Li S, and Wallis LK. (2018). Comparative toxicity of a food additive TiO2, a bulk TiO2, and a nano-sized P25 to a model organism the nematode *C. elegans*. Environmental Science and Pollution Research.

**Lenz KA**, Miller TR, and Ma H. (2018). Anabaenopeptins and cyanopeptolins induce systemic toxicity effects in a model organism the nematode Caenorhabditis elegans. *Chemosphere*, 214: 60-9.

Lu C, Svoboda KR, **Lenz KA**, Pattison C, Ma H. (2018). Toxicity interactions between manganese (Mn) and lead (Pb) or cadmium (Cd) in a model organism the nematode *C. elegans. Environmental Science and Pollution Research*, 25(3).

Sreevidya VS, **Lenz KA**, Svoboda KR, Ma H. (2018). Benzalkonium chloride, benzethonium chloride, and chloroxylenol - Three replacement antimicrobials are more toxic than triclosan and triclocarban in two model organisms. *Environ Pollut,* 235: 814-824.

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Palmersheim KA and **Lenz KA.** (2016). Adult Smoking in Wisconsin and the United States: an assessment of trends in adult smoking, 1990-2013. Milwaukee, Wisconsin: University of Wisconsin-Milwaukee, Center for Urban Initiatives and Research, Pending review by the Wisconsin Department of Health and

Family Services, Division of Public Health, Bureau of Community Health Promotion, Wisconsin Tobacco Prevention and Control Program.

Luongo S, **Lenz KA**, Palmersheim KA, and Glysch R. (2014). 2014 High School Tobacco Fact Sheet (YTS). Wisconsin Department of Health and Family Services, Division of Public Health, Bureau of Community Health Promotion, Wisconsin Tobacco Prevention and Control Program.

Luongo S., **Lenz KA**, Palmersheim KA, and Glysch R. (2014). 2014 Middle School Tobacco Fact Sheet (YTS). Wisconsin Department of Health and Family Services, Division of Public Health, Bureau of Community Health Promotion, Wisconsin Tobacco Prevention and Control Program.

**MANUSCRIPTS IN PREPARATION**

**Lenz KA** and Ma H. Insulin-like growth factor (IGF)-mediated toxicity of triclosan and triclocarban in the nematode *C. elegans*.

**Lenz KA** and Ma H. Toxicological effects of Triclosan (TCS), Triclocarban (TCC), and their degradation products to a model organism the nematode *Caenorhabditis elegans (C. elegans)*.

**AWARDS AND HONORS:**

| | |
|---|---|
| 2020 | Outstanding Graduate Student, Student Excellence Awards, University of WisconsinMilwaukee, Milwaukee, WI. |
| 2020 | Agent of Change Award, Student Excellence Awards, University of WisconsinMilwaukee, Milwaukee, WI. |
| 2020 | Exemplary Leadership Award, Student Excellence Awards, University of WisconsinMilwaukee, Milwaukee, WI. |
| 2019 | Nominee, Forbes 30 Under 30 in Science, North America, Forbes, New York, NY. |
| 2018 | Nominee, Forbes 30 Under 30 in Science, North America, Forbes, New York, NY. |
| 2018 | Third Place, Platform Presentations, Health Research Symposium, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2018 | Finalist, Point Scholar Program, Point Foundation, Los Angeles, CA. |
| 2018 | Finalist, Interprofessional Healthcare Case Competition, Milwaukee Area Team, Wisconsin Area Health Education Centers, Madison, WI. |
| 2017 | Featured Graduate Student, Public Health, UWM Report – "Graduate student research fuels UWM's culture of innovation, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2017 | Campus Inclusion Leader, Autism Self-Advocacy Network, Washington DC. |
| 2017 | Finalist, Advocates in Disability Award, HSC Foundation, Washington DC. |
| 2016-2017 | Advanced Opportunity Program (AOP) Fellowship, Graduate School, University of Wisconsin-Milwaukee, Milwaukee, WI. |
| 2016 | Student Ambassador, People to People International, Spokane, WA. |
| 2016 | Kelley Elizabeth Shea "Make Lemonade Everyday" Scholarship Recipient, Stupid Cancer, New York, NY. |
| 2016 | Shannon Yoder Award Recipient, St. Baldrick's Foundation, Monrovia, CA. |
| 2016 | Wayne Kopp Memorial Scholarship Recipient, Wisconsin Environmental Health Association, Eau Claire, WI. |
| 2015 | Nominee, Forbes 30 Under 30 in Science, North America, Forbes, New York, NY. |
| 2015 | Travel Award Recipient, Wisconsin Environmental Health Network, Madison, WI. |
| 2014-2015 | Constance A. Greiser Scholarship Recipient, University of Wisconsin-Joseph J. Zilber School of Public Health, Milwaukee, WI. |

| | |
|---|---|
| 2013 | Who's Who in American Colleges and Universities Award Recipient, St. Norbert College, De Pere, WI. |
| 2013 | Exemplary Leadership Award, Norbertine Leadership and Service Award Program, St. Norbert College, De Pere, WI. |
| 2012-2013 | Dean's List, St. Norbert College, De Pere, WI. |
| 2012 | Exemplary Leadership Award, Norbertine Leadership and Service Award Program, St. Norbert College, De Pere, WI. |
| 2011 | Exemplary Leadership Award, Norbertine Leadership and Service Award Program, St. Norbert College, De Pere, WI. |
| 2011 | Outstanding Service Organization of the Year, Disability Advocacy President, St. Norbert College, De Pere, WI. |
| 2010 | Youngest Student Organization President, Disability Advocacy President, St. Norbert College, De Pere, WI. |
| 2010 | Matthew Debono Memorial Scholarship Recipient, Aplastic Anemia and MDS International Foundation, Rockville, MD. |
| 2009-2013 | John F. Kennedy Scholarship Recipient, St. Norbert College, De Pere, WI. |
| 2009 | Wisconsin Department of Children and Families Foster Care Scholarship Recipient, Wisconsin Department of Children and Families, Madison, WI. |
| 2009 | SAMFund Grant Recipient, The SAMFund for Young Adult Survivors of Cancer, Boston, MA. |
| 2009 | Scott Deljadillo Scholarship Recipient, Friends of Scott Foundation, San Diego, CA. |
| 2009 | Bonduel Development Scholarship Recipient, Bonduel School District, Bonduel, WI. |
| 2009 | Figure 8 Muskie Club Biological and Freshwater Sciences Scholarship Recipient, Shawano County Chapter, Shawano, WI. |
| 2006-2007 | Student Ambassador, Secondary Education Delegation, People to People International, Spokane, WA. |

**PLATFORM PRESENTATIONS:**

| | |
|---|---|
| 2018 | **Lenz KA**, Miller TR, and Ma H. Anabaenopeptins and cyanopeptolins induce systemic toxicity effects in a model organism the nematode Caenorhabditis elegans. Society of Environmental Toxicology and Chemistry (SETAC) 39th Annual Meeting, Sacramento, CA. |
| 2018 | **Lenz KA**, Miller TR, and Ma H. *Anabaenopeptins and cyanopeptolins induce systemic toxicity effects in a model organism the nematode Caenorhabditis elegans.* UWM Health Research Symposium, Milwaukee, WI. |
| 2017 | Ma H, Sreevidya VS, **Lenz KA**, and Svoboda KR. Toxicological effects of three replacement antimicrobials of triclosan and triclocarban to two model organisms the nematode C. elegans and zebrafish. Society of Environmental Toxicology and Chemistry (SETAC) 38$^{th}$ Annual Meeting, Minneapolis, MN. |
| 2017 | Ma H, Sreevidya VS, **Lenz KA**, and Svoboda KR. *Comparative toxicological effects of triclosan and triclocarban to their three replacement compounds: are the replacements safer?.* International Conference on Environmental Pollution and Human Health, Jinan University, Guangzhou, China. |

**POSTER PRESENTATIONS:**

| | |
|---|---|
| 2018 | **Lenz KA**, Miller TR, and Ma H. *Anabaenopeptins and cyanopeptolins induce systemic toxicity effects in a model organism the nematode Caenorhabditis elegans.* UWM Health Research Symposium, Milwaukee, WI. |

2018 **Lenz KA**, Miller TR, and Ma H. Toxicological effects of *anabaenopeptins and cyanopeptolins in a model organism the nematode Caenorhabditis elegans. Society of Toxicology (SOT), Houston, TX.*

2017 **Lenz KA**, Pattison CR, and Ma H. *Toxicological effects of Triclosan (TCS), Triclocarban (TCC), and their degradation products to a model organism the nematode Caenorhabditis elegans (C. elegans)*. Pharmaceuticals and Personal Care Products. Society of Environmental Toxicology and Chemistry (SETAC) 38th Annual Meeting, Minneapolis, MN.

2017 **Lenz KA**, Vella K, and Andriukaitis V. The future of ecological risk management and regulatory decision-making in Europe and the North America. Society of Environmental Toxicology and Chemistry (SETAC) Europe Meeting, Brussels, Belgium.

2016 **Lenz KA**, Pattison CR, and Ma H. *Toxicological effects of Triclosan (TCS) and Triclocarban (TCC) to a model organism the nematode Caenorhabditis elegans (C. elegans)*. Terrestrial or Wildlife Toxicology and Ecology. Society of Environmental Toxicology and Chemistry (SETAC) 37th Annual Meeting, Orlando, FL.

2016 Lu C, Svoboda KR, **Lenz KA**, Pattison CR, and Ma H. *Toxicity of manganese to the nematode C. elegans in the presence of cadmium or lead.* Society of Environmental Toxicology and Chemistry, Orlando, FL.

2014 Palmersheim KA, **Lenz KA**, and Glysch R. *Tobacco has a new look: The changing face of nicotine*. American Public Health Association, Chicago, IL.

**INVITED WORKSHOPS AND CONFERENCES:**

2016 Navigating College and Cancer, CancerCon, Stupid Cancer, Denver, CO.

2016 It's All About Being a Pediatric or Teen Survivor, CancerCon, Stupid Cancer, Denver, CO.

2014 Managing Survivor Guilt, OMG! Cancer Summit for Young Adults, Stupid Cancer, Las Vegas, NV.

2012 Diversity at Private Colleges, Wisconsin Private College Initiative for Diversity (WPICD) Conference, St. Norbert College, De Pere, WI.

**INVITED LECTURES:**

2018 *How to Speak (and Listen) to Transgender and Gender Non-conforming People,* St. Norbert College, De Pere, WI.

2015 *Adolescent and Young Adult (AYA) Cancer: Before, During, and After Treatment,* Medical College of Wisconsin – Green Bay, De Pere, WI.

**INVITED SEMINARS:**

2017 *Success Beyond 18: A Better Path for Young People Transitioning from Foster Care to Adulthood*, St. Norbert College, De Pere, WI.

2015 *Beyond Class and Nation: Reframing Social Inequalities in a Globalizing World*, St. Norbert College, De Pere, WI.

**PROFESSIONAL SERVICE:**

2016-2017 US Student Ambassador, Toxicology Delegation, People to People International

2015 Liaison for the Center for Urban Initiatives and Research Invited Speakers, Paul Schmitz

2014-2016 Review Committee, University of Wisconsin System Symposium for Undergraduate Research

2014-Present Student Representative, Milwaukee Water Commons

2012-2013 Liaison for Visiting Fulbright Scholar, Mikulas Oros, PhD

2012 Liaison for the Medical College of Wisconsin Northeast Wisconsin Campus Planning and Program Review Committees
2006-2007 US Student Ambassador, European Council and People to People International

**UNIVERSITY SERVICE:**

2017-Present Campus Inclusion Leader, Autism Self-Advocacy Network, Washington DC.
2015 Session Leader, Moving Forward Together: Achieving Collective Impact, Annual Conference, Center for Urban Initiatives and Research, Milwaukee, WI.
2015 Planning Committee, Moving Forward Together: Achieving Collective Impact, Annual Conference, Center for Urban Initiatives and Research, Milwaukee, WI.
2013-Present Student Chair, Community Safety Committee, University of Wisconsin-Milwaukee, Milwaukee, WI.
2013-Present Member of the Zilber School of Public Health Student Government Association, Zilber School of Public Health, University of Wisconsin-Milwaukee, Milwaukee, WI.
2011-2013 Student Chair of Curriculum and Education Policy Committee, St. Norbert College, De Pere, WI.
2011-2013 Student Representative, Student Government Association, St. Norbert College, De Pere, WI.
2011-2012 Student Chair of Academic Honor Court, St. Norbert College, De Pere, WI.

**PROFESSIONAL MEMBERSHIPS:**

2015-Present Society of Environmental Toxicology and Chemistry
2013-Present American Public Health Association
2013-Present Wisconsin Public Health Association

**VOLUNTEER:**

2015-Present English as a Second Language Tutor, University of Wisconsin-Milwaukee, Milwaukee, WI.
2014-Present Young Adult Oncology Group Peer Support and Mentorship Program, Children's Hospital of Wisconsin, Milwaukee, WI.
2014-Present Mentor, Imerman Angels, Chicago, IL.
2012 International Medical Volunteer, Turning Responsibility into Powerful Service (TRIPS), St. Norbert College, Nicaragua.
2011 St. Jude's Volunteer, Turning Responsibility into Powerful Service (TRIPS), St. Norbert College, Memphis, TN.
2009-2013 Brown County PALS Mentor, Green Bay, WI.
2009-2011 English as a Second Language Tutor, St. Norbert College, De Pere, WI.
2008-2012 Special Olympics Volunteer, Shawano and Menominee Counties, WI.