

THE EFFECTS OF TRICLOSAN, 2,4-D, AND THEIR BY-PRODUCTS  
ON THE ADRENOCORTICAL CELLS OF RAINBOW TROUT

ANDREA B. DANN

Bachelor of Science, University of Lethbridge, 2009

A Thesis

Submitted to the School of Graduate Studies  
of the University of Lethbridge  
in Partial Fulfillment of the  
Requirements of the Degree

**MASTERS OF SCIENCE**

Department of Biological Sciences  
University of Lethbridge  
Lethbridge, Alberta, Canada

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## ABSTRACT

The ubiquitous presence of anthropogenic chemicals and their transformation products in surface water represents a toxicological concern from both an ecological standpoint and a human perspective as many of these chemicals are capable of altering hormonal function. Endocrine disrupting compounds can be traced back to numerous sources and may fall under the class of pesticide, industrial chemical, pharmaceutical, personal care product, and/or heavy metals. The adrenal gland is the most common target for endocrine disruptors, although in comparison to the sex steroids, this system has received much less attention in published research. Corticosteroids play a pivotal role in many physiological processes, including immunity, cognitive function, growth, metabolism, reproduction, mineral balance, and blood pressure. A primary cell culture of rainbow trout adrenocortical cells was used to investigate the endocrine disrupting activity of two commonly detected water-borne toxicants, a personal care product, triclosan (TCS), a pesticide, dichlorophenoxyacetic acid (2,4-D), and their transformation products, methyl-triclosan (M-TCS) and dichlorophenol (DCP). Previously, it has been shown that TCS, 2,4-D, and DCP exhibit a potential for endocrine disruption, although it is currently unknown if these chemicals are capable of affecting corticosteroid balance. In this study, all four chemicals showed significant inhibitory effects on corticosteroid synthesis, even though there were considerable differences in their activity. The chemical that exhibited the highest toxicity was 2,4-D, followed by TCS, DCP, and M-TCS. Both parent-compounds proved to be more toxic than their degradation products. More research with suitable test systems is needed to determine the mechanism(s) of action of these corticosteroid disruptors and the health risk that they may present.

## **ACKNOWLEDGEMENTS**

Firstly, I would like to thank my supervisor, Dr. Alice Hontela for all of her support and guidance on this project. My interest in toxicology was ignited in one of Dr. Hontela's physiology classes and it was due to her encouragement that I chose to pursue an M.Sc degree. I would like to convey my sincerest gratitude for her criticisms and insights pertaining to this thesis. I would like to acknowledge Roger Royer for all his hard work maintaining the aquatic facility and assisting with the animal husbandry. I would like to express appreciation to my academic supervisory committee members, Dr. Andrew Iwaniuk and Dr. Roy Golsteyn for their suggestions and feedback. Lastly, I would like to recognize the generous assistance received from my family. I could not have finished the thesis without their support and cannot hope to adequately thank them. This project was funded by the University of Lethbridge and NSERC.

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# CHAPTER 1. TRICLOSAN, 2,4-D, AND THEIR BY-PRODUCTS: KNOWLEDGE GAPS AND RESEARCH OBJECTIVES

## 1.1 Introduction

There exists a number of different pathways for anthropogenic chemicals to enter the aquatic environment. The primary source(s) of water-borne toxicants include municipal and industrial wastewater, non-point source runoff and atmospheric deposition (Campbell et al., 2006). Once in the aquatic environment, chemicals may undergo a number of different degradation processes to produce by-products (Lin et al., 2006). Water-borne chemicals are widely distributed in surface water around the world and are typically detected in the ng l<sup>-1</sup> to µg l<sup>-1</sup> range. Many classes of chemicals, such as pharmaceuticals, pesticides, industrial chemicals, and phytochemicals have a ubiquitous presence in most rivers and streams (Kolpin et al., 2002). Accordingly, fish and other aquatic biota are chronically exposed to varying levels of potentially biologically active water-borne toxicants. Many of these chemicals are capable of interfering with the endocrine systems of non-target species. Endocrine disrupting compounds (EDCs) represent a class of environmental toxicants that interfere with synthesis, secretion, transport, binding, action, or elimination of endogenous hormones (Crisp et al., 1998). Chlorinated insecticides, herbicides, PCB's, plasticizers, and xenoestrogens have all been shown to alter steroidogenesis, the biosynthesis of steroid hormones (Andersen and Barton, 1999; Matozzo et al., 2008). More research is needed to determine the effects of complex mixtures of water-borne toxicants on non-target species, particularly fish.

The most common target organ for endocrine disruption is the adrenal gland (Harvey et al., 2007). Until recently, the bulk of research on how chemicals in the environment alter hormone function has been narrowly focused upon the sex steroids. The number of toxicants that interfere with the function of the adrenal gland is extensive, ranging from heavy metals (Lacroix

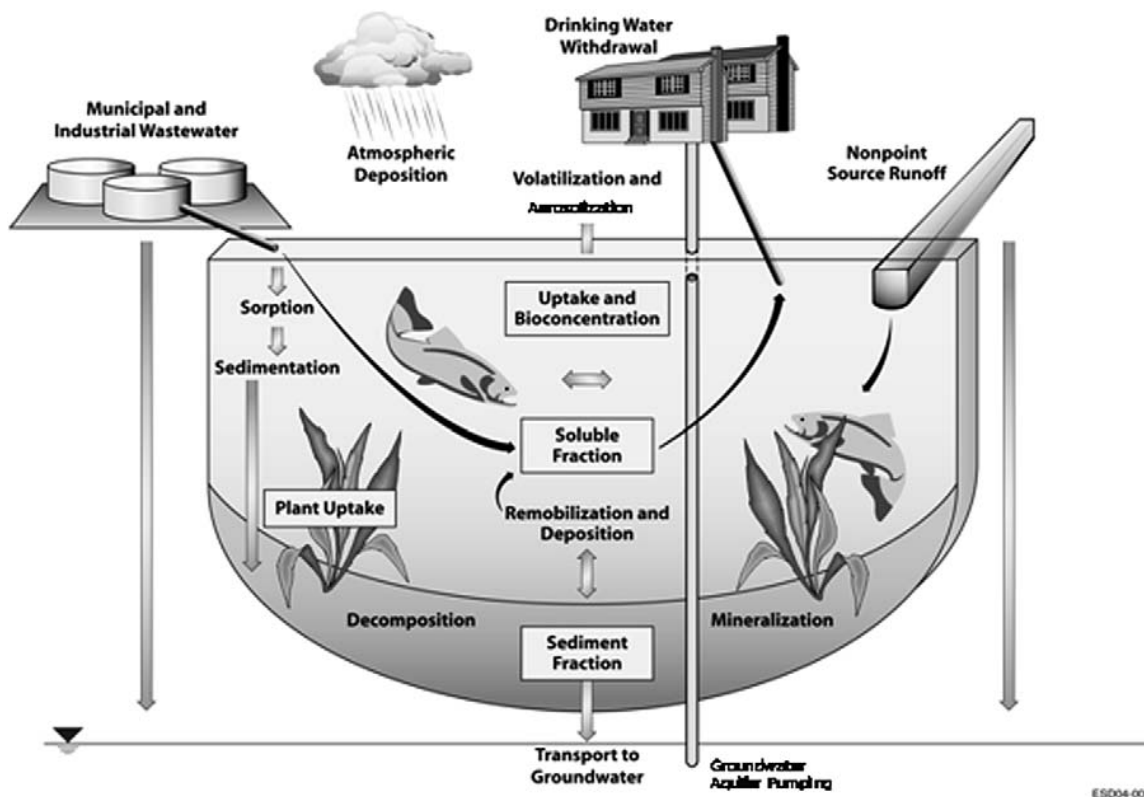


Figure 1.1. Schematic representation of the different processes and compartments that need to be monitored to characterize the fate and transport of EDCs in the environment (adapted from Campbell et al., 2006).

and Hontela, 2004) to organochlorides (Brogan et al., 1984), to aromatic hydrocarbons (Engelhardt et al., 1991). To investigate adrenal toxicity in vitro, the H295 adrenocortical carcinoma cell line is commonly studied and is particularly helpful for the isolation of molecular targets for steroidogenic disruption. Corticosteroid production occurs in the cortex of the adrenal gland, or the interrenal cells of fish and amphibians. The biosynthesis of cortisol is regulated by adrenocorticotropin hormone (ACTH), which is released from the anterior pituitary under the control of the hypothalamus, via corticotrophin releasing hormone (CRH) (Mommsen et al., 1999). In fish, corticosteroids play an important role in mediating the stress response, but are also essential for the regulation of growth, reproduction, immune function, osmoregulation, cell proliferation and differentiation (Mommsen et al., 1999). The importance of corticosteroids in biological processes must not be underestimated. Abnormal levels of corticosteroids are

implicated in a number of diseases, including osteoporosis, cataract formation, obesity, type 2 diabetes, cardiovascular, inflammatory, and autoimmune disease (Carnahan and Goldstein, 2000; Cooper 2004; Freel and Connell, 2004; Rosmond 2005).

While the adrenal gland of mammals is identical in function to the cortisol producing interrenal cells of teleost fish, important structural differences do exist. Adrenal tissue in mammals is situated in a compact, highly vascularized gland, whereas in fish and amphibians, corticosteroid producing cells are diffusely located in the interrenal tissue (Hinson and Raven, 2006). Based on these structural differences, it would seem that in order to most accurately assess the impact of water-borne toxicants on the biosynthetic pathways of cortisol production in fish, a fish cell culture should be favoured over more frequently used mammalian cell lines, including the H295 cells. According to Mommsen et al. (1999), researchers should refrain from using mammalian models to explain the stress response in fish, because the differences between mammals and fish are too great to allow for such generalizations. A primary cell culture of rainbow trout interrenal cells was developed by Leblond and Hontela (1999), for a species specific model for use in endocrine toxicology testing. This model has been used successfully to screen for a number of different chemicals that target the HPI (hypothalamus-pituitary-interrenal) axis (Goulet and Hontela, 2003; LeBlond and Hontela, 1999; Hontela and Vijayan, 2009). Fish cultures of interrenal cells constitute an excellent tool for the screening of EDCs, in addition to being well-suited for studies seeking to elucidate the molecular mechanisms of HPI toxicity. In the present study, the potential of a personal care product (Triclosan, TCS), an herbicide (2,4-D) and their by-products, to disrupt the synthesis of cortisol, the corticosteroid hormone of teleost fish, will be assessed *in vitro* using the primary culture of rainbow trout interrenal cells.

## **1.2 Triclosan**

### *1.2.1 Properties and Sources*

Triclosan [TCS; (5-chloro-2-(2,4-dichlorophenoxy)phenol)], a halogenated phenol, is a commonly used antimicrobial that is incorporated into dish soap, detergent, toothpaste, mouthwash, hand soap, fabric, deodorant, and shampoo, in addition to innumerable other personal care and household products (see Chapter 2, review by Dann and Hontela, 2011). TCS is the generic name for the chemical and brand names include Irgasan DP300, Aquasept, Gamophen, Sapoderm, and Ster-Zac (Merck Index, 2006). TCS is lipophilic, as evidenced by its octanol/water partition coefficient of 4.8 (Adolfsson-Erici et al., 2002), giving it the potential to bioaccumulate in aquatic species. Most of the products containing TCS are washed down residential drains, eventually entering waste water treatment plants (WWTP). As such, there is concern about the fate of TCS during the sewage treatment process. This concern is well warranted, as TCS is one of the most frequently detected organic wastewater contaminants in North America, having been detected in 57.6% of US streams and rivers (Kolpin et al., 2002). TCS's presence in surface water is ubiquitous around the world (Kolpin et al., 2002; Bester, 2005; Nakada et al., 2008).

### *1.2.2 Occurrence in the Aquatic Environment*

In samples collected from a water treatment facility in Ontario, influent concentrations of TCS ranged from 0.01-4.01  $\mu\text{g/L}$ , with corresponding effluent concentrations being reduced to 0.01-0.324  $\mu\text{g l}^{-1}$ . According to this particular study, the reduction of TCS ranged from 74% to 98% (Lishman et al., 2006). These results are consistent with tests conducted at several WWTPs in Switzerland and Germany, where 4% to 10% of TCS remained dissolved in out-flowing water (Singer et al., 2002; Bester, 2003). Field measurements from a Swiss WWTP have detailed the elimination process of TCS; 79% was biologically degraded, 15% was sorped to sludge, and 6% left the plant in the final effluent at a concentration of 42  $\text{ng l}^{-1}$  (Singer et al., 2002). Levels of TCS in Canadian surface waters (Servos et al., 2007) are similar to those reported elsewhere in the world.

### 1.2.3 Environmental Behavior of Triclosan

The environmental fate of TCS is not completely understood, although during the sewage treatment process, a small percentage of TCS is methylated to form Methyl-Triclosan (M-TCS). Approximately 20-30% of TCS that is released from WWTPs is converted into M-TCS (Bester 2005). However, in addition to M-TCS, the photodegradation of TCS produces several toxic transformation products. These include 2,7/2,8-dibenzodichloro-p-dioxin, toxic chlorophenols; 2,4-dichlorophenol and 2,4,6-trichlorophenol, chlorophenoxyphenols; 5,6-dichloro-2-(2,4-dichlorophenoxy)phenol, 4,5-dichloro-2-(2,4-dichlorophenoxy)phenol, and 4,5,6-trichloro-(2,4-dichlorophenoxy)phenol, an unknown radical compound with the formula  $C_{12}H_4O_3Cl_4$ , and chloroform (Canosa et al., 2005; Rule et al., 2005; Greynock and Vikesland, 2006; Fiss et al., 2007; Vanderford et al., 2008). The most stable by-products of TCS are 2,4-dichlorophenol and 2,4,6-trichlorophenol, which have been measured along with TCS in several raw waste water samples (Canosa et al., 2005). Direct photolysis of TCS is responsible for the production of 2,7/2,8-dibenzodichloro-p-dioxin, while reactions with chlorine lead to the formation of chlorinated phenols.

### 1.2.4 Effects in Animals

Triclosan's ability to act both estrogenically and androgenically, along with its potential to mimic thyroid hormones, is enough to warrant further research on its endocrine disrupting effects in both aquatic species and humans. Unfortunately at the present time, data on TCS and endocrine disruption in fish is scarce (see Chapter 2, review by Dann and Hontela, 2011), with the exception of a small number of studies that investigated the estrogenic/androgenic effects of the compound in Japanese Medaka (*Oryzias latipes*).

It is apparent that further studies are needed to confirm TCS's endocrine disrupting effects, especially in aquatic animals, which are chronically exposed to low levels of the chemical and its metabolites. Specifically, more research is needed to fully comprehend the endocrine disrupting effects of TCS in fish species whereby endocrine disruption parameters are not solely

focused on reproductive function. In general, more information on the adverse effects, mechanisms of actions, and risk potential of TCS is needed, in order to ensure that aquatic ecosystems are sufficiently protected from any adverse effects the chemical may produce. A comprehensive review of the aquatic toxicity of TCS is presented in Chapter 2 of this thesis (Dann and Hontela, 2011).

### **1.3 2,4-D**

#### *1.3.1 Properties and Sources*

In 2007, the Canadian pesticide market was valued at an impressive 1.424 billion dollars, representing a slight increase over sales in 2006 (Crop Protection, 2008). Herbicides account for the majority of pesticides sold in Canada, constituting 75% of all pesticide sales in 2007 (Brook, 2008). In 2003, 9 264 487.7 kg of pesticide active ingredients were sold or shipped into Alberta (Byrtus, 2007). Of these active ingredients, phenoxy acids (primarily 2,4-D) accounted for 21.1% of all pesticide sales in Alberta which translates into 763 357.6 kg of 2,4-dichlorophenoxyacetic acid (2,4-D) across all sectors (agricultural, domestic, and industrial). Between 1998 and 2003, 2,4-D sales increased by 1.5 % which serves to reinforce the pesticide's longstanding relevance in agricultural sectors. 2,4-D, a broad leaf phenoxy herbicide, is one of the most commonly used pesticides in North America, if not globally, and is frequently detected in surface water and air. This is unremarkable considering that in 1990, more than 3.8 million kg of 2,4-D were applied in Alberta, Saskatchewan, and Manitoba alone (Waite et al., 2002). 2,4-D was introduced into the commercial pesticide market in the 1940's (Bus and Hammond, 2007) and has enjoyed widespread market success ever since. It is a versatile pesticide that can be used in wide variety of settings including agriculture crops, forestry, turf, non-crop, and aquatic weeds (Bus and Hammond, 2007).

2,4-D exists in one of three forms: acid, 2-ethylhexyl ester, or dimethylamine salts (Bus and Hammond, 2007). Henceforth, any reference to "2,4-D" with no specifications will include all three of the chemical forms. The phenoxy acid family of pesticides disrupts plant cell growth

in newly formed areas of the plant; affecting protein synthesis and mitosis thereby leading to dysfunctional growth and tumors (Brook, 2008). Regardless of whether the pesticide was applied as an ester or amine salt, it is hydrolyzed to the acid form in the environment (Waite et al., 2002). 2,4-D amine salts are nonvolatile and are considerably more water soluble than the acid whereas the low-volatile esters are more volatile but less water soluble than the acid (Waite et al., 2002). The log octanol/water partition coefficient of 2,4-D is 2.81 indicating that the chemical is slightly lipophilic (Howard, 1991).

### *1.3.2 Occurrence in the Aquatic Environment*

When pesticides are applied to a given area, some degree of movement away from the target site is likely to occur and should be expected, although the applicator must attempt to minimize migration. There are several ways in which pesticides can migrate away from their target site, including dissolution in runoff water and subsequent transportation to surface and/or groundwater, or volatilization into the atmosphere (Kumar, 2001). Once pesticides enter the environment, they can travel significant distances before they are deposited in land or water. Agriculture and Agri-Food Canada determined that 2,4-D is the principal pesticide detected in rainfall in southern Alberta (Anderson, 2005). The extent to which a given pesticide will be detected in land, water, or air depends on the log octanol water partition coefficient (propensity to bioaccumulate), organic carbon partitioning coefficient (adsorption to soil), hydrophilicity (solubility in water), and Henry's Law Constant (volatilization) (Byrtus et al., 2002). In water, a certain percentage of the herbicide will be degraded by photolysis, hydrolysis, or other degradation pathways (Borges et al., 2004).

In Alberta, 2,4-D is the most commonly detected pesticide in surface water, with a detection rate of 53% (Anderson, 2005), a rate which is particularly high in the south, where agriculture is most practiced. One might assume that pesticides would be detected in river systems primarily during their period of application (May-July period). This, however, is not the case. Pesticides, including 2,4-D, are detected year round and surprisingly enough,

concentrations do not peak during the application period (Anderson, 2005). Intense agriculture surrounding the Oldman river basin resulted in the river system receiving the highest frequency of pesticide detection in Alberta, with a rate of 46.9%.

When comparing pesticide concentrations in untreated and treated water, data from water treatment processes in Lethbridge and Carmangay indicate that water treatment processes are unable to effectively remove pesticides (Anderson, 2005). From this data, it can then be concluded that water treatment processes in Alberta are not always effective in ensuring the removal of pesticide residue(s). It is most certainly no coincidence that 2,4-D is the most commonly detected pesticide in surface water and precipitation in southern Alberta, a fact which is mirrored by detection rates closely paralleling the sales and use of the pesticide in this region.

Concerns have been raised about the large volumes of 2,4-D that are currently used in agriculture, especially since numerous researchers have voiced concerns that 2,4-D may carry reproductive risks and/or pose mutagenic and carcinogenic risks. Epidemiological studies have provided equivocal evidence that 2,4-D is a possible carcinogen, while any such links have not been proven in laboratory studies (Garabrant and Philbert, 2002). Widespread concern over the use of 2,4-D is probably linked to 2,4-D's connection with Agent Orange, which was a 50:50 mixture of 2,4-D and 2,4,5-T that was tainted with dioxin. Epidemiological studies may have provided inconclusive results because 2,4-D mixtures may exhibit varying degrees of TCDD contamination (Masunaga et al., 2001). In addition, pesticide applicators are often exposed to numerous different chemicals, making it difficult to link a specific chemical with an increased risk for cancer.

### *1.3.3 Environmental Behaviour*

2,4-D enters the environment primarily through agricultural applications, although commercial and domestic applications represent minor pathways for environmental contamination. 2,4-D has been detected in air, surface water, and ground water in Canada (Waite



et al., 2002). The migration (drift and runoff) of pesticides from their area of application to groundwater and surface water is a common problem. In soil, the biodegradation of 2,4-D is the single most important elimination pathway for the chemical (Howard, 1991). The degradation of 2,4-D in soil is a rapid process, with the chemical having a half-life of 6.2 days on average (USEPA, 2005). However, the elimination of 2,4-D from aquatic ecosystems is a considerably slower process.

The functional groups of 2,4-D make the chemical somewhat resistant to hydrolysis, but as the pH of the water increases, the chemical becomes more hydrophilic, especially at pH's greater than 8 (Howard 1991). 2,4-D esters are rapidly hydrolyzed in alkaline conditions where 2,4-D amine salts have the propensity to dissociate in water. Under a wide array of environmental conditions, 2,4-D amine and esters will degrade swiftly to form 2,4-D acid (USEPA, 2005). Degradation products of 2,4-D acid include 1,2,4-benzenetriol, 2,4-dichlorophenol (2,4-DCP), 2,4-dichloroanisole (2,4-DCA), chlorohydroquinone (CHQ), 4-chlorophenol, volatile organics, bound residues, and carbon dioxide (Borges et al., 2004). According to the US Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP), the degradation products of 2,4-D are not considered to be an environmental or health concern and as such, are not included in ecological risk assessments (Borges et al., 2004).

The rate of 2,4-D breakdown in aquatic environments depends heavily on dissolved oxygen concentrations in the water. The chemical degrades rapidly in well oxygenated waters, with a half-life of 15 days, whereas anaerobic aquatic environments result in 2,4-D persistence, with half-life of 41 to 333 days (USEPA, 2005). 2,4-D esters are volatile, especially under conditions of low humidity and high temperatures. Generally speaking, 2,4-D does not adhere to sediment and is frequently detected in surface and ground water. Biological, chemical, and photodegradation all play a role in the environmental fate of 2,4-D in aquatic ecosystems (Waite et al., 2002).

#### *1.3.4 Effects of 2,4-D in Animals*

2,4-D is soluble in water, distributing widely through aquatic environments and having a tendency to persist over time. The pesticide can act as a direct toxicant to fish, but may also act indirectly when fish consume invertebrates or aquatic plants contaminated with 2,4-D (Borges et al., 2004). In the body, 2,4-D exists primarily in the ionized form and active transport is required in order for it to pass through the lipid bilayer of cells (Garabrant and Philbert, 2002). Excretion occurs primarily through renal elimination, whereas the dose of the chemical increases, metabolite excretion in feces becomes a more important elimination pathway (Garabrant and Philbert, 2002). 2,4-D acids and salts are practically non-toxic to slightly toxic to fish, whereas the ester form has proven to be slightly toxic to highly toxic to freshwater fish species (Borges et al., 2004). Specifically, 2,4-D ester formulations in 96-hour acute toxicity tests with freshwater fish resulted in an LC<sub>50</sub> that was three magnitudes greater than the other forms. However, it is important to note that the “inert ingredients” in commercial pesticide formulations of 2,4-D could affect its toxicity, either additively, synergistically and/or antagonistically.

The estrogenic potential of 2,4-D and a binary mixture of the herbicide with one of two surfactants (R-11 and Target Prospread Activator) was examined in juvenile rainbow trout (Xie et al., 2005). Juvenile rainbow trout that were exposed to 2,4-D for a period of 7 days had a 93- fold increase in plasma vitellogenin levels, compared to untreated fish. When the fish were exposed to the binary mixtures, the estrogenic effects of 2,4-D were enhanced. The binary mixture of alkylphenol ethoxylate-containing surfactants with 2,4-D resulted in greater than additive estrogenic effects in fish, as evidenced by marked increases in vitellogenin production. As previously mentioned, one of the degradation products of 2,4-D in water is 2,4-dichlorophenol, which can bind to estrogen receptors in fish, stimulating vitellogenin production in male and/or juvenile fish (Jobling et al., 1995). Currently, this is one of the only studies in the literature that has examined the endocrine disrupting effects of pesticides with surfactants.

An *in vitro* study by Orton et al. (2009) using a recombinant yeast screen assay failed to detect any receptor mediated (anti-)estrogenic and (anti-) androgenic activity for 2,4-D. Previous studies on 2,4-D and gonadal steroids have failed to provide evidence of any direct estrogenic activity (Nishihara et al., 2000; Seifert et al., 1998; Crain et al., 1997). However, when combined with testosterone, 2,4-D has androgenic synergistic effects (Kim et al., 2005). Despite the lack of clear evidence linking 2,4-D to alteration in steroid hormone activity, the phenoxy herbicide elevates LH levels in humans (Garry et al., 2001).

Gonadal steroids are not the only target for 2,4-D induced endocrine disruption. Pathology data in a subchronic study on the toxicity of the three forms of 2,4-D in rats showed alterations in the testicular tissue, the adrenal cortex, and the thyroid gland (Charles et al., 1996). The exposure of teleosts and mammals to 2,4-D decreases thyroxine (T<sub>4</sub>) content in the thyroid gland and lower serum levels of both triiodothyronine (T<sub>3</sub>) and T<sub>4</sub> (Kobal et al., 2000; Raldua and Babin, 2009). Rats exposed to all three forms of 2,4-D exhibited a decrease in thyroid weight and a concomitant decrease in circulating thyroid hormone levels (Charles et al., 1996). Lastly, in a reporter gene assay for pregnane X receptor agonist activity, 2,4-D possessed PXR-mediated transcriptional activity (Kojima et al., 2011). The ability of 2,4-D to mediate PXR transcriptional activity could potentially explain why the herbicide can alter steroid hormone production, as the induced proteins play a role in steroid hormone catabolism.

#### *1.3.5 Commercial Pesticide Formulations*

Surfactants, solvents, and other ingredients consist of what is referred to as “inert” ingredients in pesticide formulations. The term “inert ingredients” is incredibly misleading to consumers, because it implies that the ingredients are safe and/or inactive, when the reality is they may be more toxic than the active ingredient itself. In addition, these “inert” ingredients may be present in large amounts, constituting up to 99% of certain pesticide formulations. To further complicate things, many pesticide formulations contain several active ingredients in addition to mixtures of “inert” ingredients. When the complex composition of some pesticide

formulations is taken into consideration, it soon becomes apparent that toxicity testing using commercial formulations of 2,4-D mixtures is the most environmentally relevant way to assess the pesticide's effects on aquatic organisms.

Comparing the toxicity of commercial pesticide mixtures to the active ingredient alone is of considerable importance in toxicology because it allows for the detection of any additive, synergistic, or antagonistic effects that the formulated product may have. This approach is practical and more efficient than testing individual chemicals for two reasons: 1) getting information on the inert ingredients in a pesticide formulation is a difficult process as chemical companies claim they are "trade secrets", a situation which is compounded by a lack of transparency in government agencies that regulate pesticide use; and 2) testing individual inert ingredients is time consuming and would not include the sum of the effects of all ingredients (Borges et al., 2004). Unfortunately, data on the aquatic toxicity of various pesticide formulations of 2,4-D is scant. In order for pesticide research to be considered environmentally relevant, toxicological studies on commercial formulations of 2,4-D must be conducted. If future studies do not include commercial formulations, interactions between the active ingredient(s) and the "inerts" may go undetected.

## **1.4 Dichlorophenol (DCP)**

### *1.4.1 Properties and sources*

In the aquatic environment, both TCS and 2,4-D undergo degradation processes that yield the phenolic by-product, DCP (CAS: 120-83-2). The molecular formula for DCP is  $C_6H_4C_{12}O$ , with the chemical existing in the form of a white solid and carrying a strong medicinal odour (HSDB, 2009). Its molecular weight is 163.00, and with a log octanol water coefficient of 3.06, the chemical can be expected to display a slight to moderate potential for bioaccumulation. Dichlorophenol is used to manufacture a number of different commercial products such as the phenoxy herbicides, 2,4-D and 2,4,5-T, dyes, mothproofing, antiseptics, seed disinfectants, wood preservatives, antihelminthic agents, and is a major waste product produced by pulp and

paper mills (HSDB, 2009). The use of DCP in the synthesis of numerous chemicals provides a pathway of entry for the chemical into the aquatic environment, although it is the degradation of 2,4-D that is the primary source of the DCP (HSDB, 2009) .

#### *1.4.2 Occurrence in the Aquatic Environment*

The detection frequency of DCP in surface water is similar to those of its parent compound(s). In fact, DCP is one of the most commonly detected chlorophenols in the aquatic environment (House et al., 1997). Surface water levels of DCP have been reported up to a maximum concentration of 19.96  $\mu\text{g l}^{-1}$  (Gao et al., 2008). Both the U.S. EPA and the European Union have flagged DCP as a priority pollutant in effluent and the aquatic environment.

DCP in surface water has been measured in China (n.d.-19.96  $\mu\text{g l}^{-1}$ ) (Gao et al., 2008), France (n.d.- 4.72  $\mu\text{g l}^{-1}$ ) (Chiron et al., 2007), Greece (n.d.-6.11  $\mu\text{g l}^{-1}$ ) (Dimou et al., 2006), Spain (1-10  $\mu\text{g l}^{-1}$ ) (Brossa et al., 2005), England (n.d.- <1.0  $\mu\text{g l}^{-1}$ ) (House et al., 1997), and Canada (n.d.- 1.58  $\mu\text{g l}^{-1}$ ) (Anderson, 2005).

#### *1.4.3 Environmental Behaviour*

In the aquatic environment, DCP will absorb to particulate matter and sediment, due to its octanol-water partition coefficient ( $K_{ow}$ ) value of 3.06 (Hansch et al., 1995; HSDB, 2009). The estimated river and lake half-lives of DCP are 14 and 103 days, respectively (HSDB, 2009). Water analysis is not the only way to monitor the prevalence of chlorophenols in the aquatic environment. It is known that chlorophenols are metabolized and excreted in the bile of fish (Al-Arabi et al., 2005) and fish bile and/or whole fish samples are frequently used as biomonitoring tools for the determination of fish exposure to water-borne chemicals. DCP has been measured in the bile of Nile tilapia (*Oreochromis niloticus*) and sharp tooth catfish (*Clarias gariepinus*) from a Bangladeshi river (Al-Arabi et al., 2005), the bile of perch (*Perca fluviatilis*) collected from the Baltic Sea (Soderstrom et al., 1994), and in whole fish samples collected from Lake Michigan tributaries (Camanzo et al., 1987).

The first researchers to investigate the bioconcentration of DCP were Kishino and Kobayashi (1996). At a concentration of 0.5 mg l<sup>-1</sup>, over pH ranges of 6, 8, and 10, the bioconcentration factor (BCF) of DCP in goldfish (*Carassius auratus*) was 40, 33, and 2.5, respectively. A later study by Kondo et al. (2005) determined the bioconcentration factor for environmentally relevant levels of DCP in Japanese medaka. Following an exposure period of 60 days, at concentrations between 0.3-30 µg l<sup>-1</sup>, BCF values were reported as the following: 3.4x10<sup>2</sup> at 0.235 µg l<sup>-1</sup> up to 92 at 27.3 µg l<sup>-1</sup>. These values indicate a low to moderate bioconcentration potential for DCP.

#### 1.4.4 Effects in Animals

For fish, the LC<sub>50</sub> value of DCP ranges from 7.0 to 11.6 mg l<sup>-1</sup> (Phipps et al., 1981), with a no observed effect concentration (NOEC) of 3.3 mg l<sup>-1</sup> in medaka (Kondo et al., 2005). Zhang et al. (2008), exposed rare minnows (*Gobiocypris rarus*) to various concentrations of DCP for 3 and 21 day. At concentrations at or above 0.3 mg l<sup>-1</sup> of DCP, toxicity and endocrine disruption were observed in both exposure periods. In female fish, Vtg expression (mRNA) and protein was significantly increased following a 21-d exposure period, while the expression of hepatic ER β mRNA was downregulated. In male fish, the opposite phenomenon was observed; protein and mRNA Vtg levels were not affected, while the expression of hepatic ER β was upregulated. The reason for these sex differences is not known. During both exposure periods, the GSI of male and female fish was reduced. Due to abnormal gonadal histopathology, it is possible that the decrease in GSI could be the result of sex tissue degeneration.

Based on the chemical structure of DCP, there is reason to suspect that the phenol may be an endocrine disruptor. Although DCP is suspected of having estrogenic activity, results from *in vitro* receptor binding tests are equivocal. Studies by Jobling et al. (1995), Korner et al. (1998), and Kramer and Giesy (1999), failed to uncover an estrogenic potential for the phenol. On the other hand, studies by Jones et al. (1998), Nishihara et al. (2000), and Han et al. (2002) reported estrogenic activity for DCP, and although these findings were statistically significant,

the magnitude of the activity was weak, and their biological relevance questionable. Building on the findings from previous *in vitro* studies, an *in vivo* study was conducted by Aoyama et al. (2005) to profile the two-generation reproductive effects of DCP in Wistar-Hannover rats. In the rats who received DCP in their diets, implantation sites and live births were decreased, whereas uterine weights for F<sub>1</sub> and F<sub>2</sub> weanlings increased significantly. This *in vivo* study provides evidence of DCP reproductive toxicity, and although the mechanism of the toxicity is not known, it is possible that these effects can be attributed to an estrogenic potential of the chemical.

In mammalian studies, when combined with dihydroxytestosterone (DHT), DCP stimulates AR transcriptional activity in human prostate cancer cell lines (Kim et al., 2005). A previous study by the same authors demonstrated that an oral dose of DCP alone (100 mg kg<sup>-1</sup> day<sup>-1</sup>) increased the weights of androgen dependent tissue, and in the presence of testosterone (1 mg kg<sup>-1</sup>), these increases became synergistic in nature (Kim et al., 2002). The results of the previous two studies were confirmed by Li et al. (2010), whose *in vitro* reporter assay test system established that DCP is an AR antagonist, inhibiting  $\beta$ -galactosidase activity in a dose-dependent fashion.

There is a growing body of evidence to suggest that DCP acts as an AR antagonist. The ubiquitous presence of DCP in the aquatic environment is of concern, as AR antagonists negatively impact normal male development. Kiparissis et al. (2003) demonstrated that the exposure of fish to anti-androgens is associated with gonadal abnormalities, specifically the induction of intersex, decreased spermatogenesis, and lower sperm counts. The long-term effects of fish exposure to AR antagonists are unknown, although when coupled with xenoestrogen exposure, the effect on fish reproduction could be profound.

### **1.5 Methyl-Triclosan (M-TCS)**

### *1.5.1 Properties and Sources*

In WWTPs, TCS undergoes biological methylation to produce its primary by-product, M-TCS. The levels of M-TCS that discharged from WWTPs into receiving waters are equal to approximately 2% of outflowing TCS (Poiger et al., 2003), with M-TCS levels increasing after water treatment processes. Once TCS has undergone methylation, its lipophilicity increases. The octanol-water partition coefficient of M-TCS is 5.4, compared to 4.76 for the parent compound, indicating that M-TCS is more lipophilic and potentially more environmentally persistent.

### *1.5.2 Occurrence in the Aquatic Environment*

M-TCS levels have been measured in surface in water in Canada (Andressen et al., 2007), Germany (Bester, 2005), and Switzerland (Poiger et al., 2003; Lindstrom et al., 2002; Balmer et al., 2005), with levels ranging from n.d.-10 ng l<sup>-1</sup>. M-TCS levels in effluent range between n.d.-20 ng l<sup>-1</sup> (Coogan et al., 2007; Bester, 2005). While concentrations of TCS in influent are far greater than in effluent, M-TCS exhibits the reverse pattern, supporting the fact that during wastewater treatment processes, TCS undergoes biological methylation to yield M-TCS.

Concentrations of M-TCS measured in semi-permeable membrane devices are magnitudes higher than concentrations typically measured in surface water, indicating that M-TCS is likely to accumulate in aquatic organisms. The tendency for M-TCS to accumulate in semi-permeable membrane devices suggests that M-TCS will bioaccumulate in aquatic organisms. M-TCS levels in fish tissue range from n.d.-2800 µg kg<sup>-1</sup> per wet weight basis (Leiker et al., 2009; Buser et al., 2006; Balmer et al., 2004), in caged snails downstream from a WWTP, with levels reaching 49.8 ng g<sup>-1</sup> (Coogan and La Point, 2008), and in algae at concentrations upwards of 89 ng g<sup>-1</sup> (Coogan et al., 2007; Coogan and La Point 2008). Based on the levels of M-TCS measured in algae and snails, the bioaccumulation factor of the chemical is 1,200, a factor of approximately three orders of magnitude.



### *1.5.3 Environmental Behaviour of M-TCS*

Evidence to support a higher bioaccumulation potential for M-TCS comes from a study by Lindstrom et al. (2002) who measured M-TCS in passive semi-permeable sampling devices, but failed to detect any presence of the parent compound. Both Lindstrom et al. (2002) and Balmer et al. (2004) demonstrated that TCS is photolabile, while M-TCS is relatively unaffected by photolysis. Sorption and sedimentation are expected to play a role in the removal pathway of M-TCS from aquatic environments (Lindstrom et al., 2002). The estimated half-life of M-TCS in river systems is at least 11 days (Bester, 2005), with removal rates varying considerably, depending on the individual characteristics of an aquatic system.

According to Boehmer et al. (2004), M-TCS is a persistent organic pollutant commonly detected in surface water, and little is known about its aquatic toxicity. Future studies are needed to determine what, if any, ecological impact M-TCS is having, or will have on aquatic ecosystems.

## **1.6 Mixtures**

The endocrine disrupting potential of environmental contaminants is a relevant area of research, with many studies reporting that these chemical mixtures are affecting the estrogen receptor (ER) in cells and subsequently interfering with normal hormone function (Ahn et al., 2008). To a lesser degree, researchers have begun to address the presence of androgenic chemicals in the environment, which can cause decreases in spermatogenesis and genital abnormalities (Ahn et al., 2008). Considering that the adrenal gland is extremely vulnerable to endocrine disruption, it is surprising that the bulk of research on endocrine disruption has focused disproportionately on the estrogenic and anti-androgenic effects of water-borne toxicants. To further complicate things, it is highly implausible that fish in river systems would ever be exposed to only one chemical. Aquatic environments almost always contain complex mixtures of chemicals including pharmaceuticals, pesticides, metals, surfactants, and personal care products, many of which may exhibit an endocrine disrupting potential.

Investigation of endocrine disruption in aquatic ecosystems necessitates a ‘real world’ approach, where fish are exposed to mixtures of environmental contaminants rather than individual chemicals. Studies conducted by Sumpter and Jobling (1995) suggested that fish living in an estrogenic milieu might exhibit more pronounced endocrine disruption when exposed to a mixture of estrogenic chemicals, than if they were exposed to just a single estrogenic chemical at the same concentration. The synergistic potential of mixtures of environmental contaminants should not be underestimated, as the interplay between chemicals can elicit responses from biological systems that might be greater or less than what would be anticipated.

For urban centers, input from WWTP into river systems downstream can be significant, especially in periods of low rainfall, so much so that entire river systems may become estrogenic (Sumpter, 1998). With climate change, water scarcity in semi-arid regions such as Southern Alberta will almost certainly result in higher concentrations of contaminants in aquatic environments, potentially increasing their endocrine disrupting effects. Many studies focused on Vtg induction in male fish as a biomarker of estrogen exposure, and Kidd et al. (2007) provided key evidence, linked to Vtg induction, about population collapse in fathead minnow. Other endocrine disruption parameters such as disruption to the adrenal and/or thyroid axis have not been sufficiently researched. Specifically, more research is needed to profile the magnitude of the endocrine disrupting potential of chemicals that are commonly detected in river systems, to what extent these chemicals may bioaccumulate in fish species, and what the consequences of chronic exposure may be. Fish are an excellent choice of sentinel species for the assessment of water quality, especially now that the general public is becoming cognizant that human and environmental health are inextricably linked.

## **1.7 Physiological Stress Response**

### *1.7.1 Hypothalamic-Pituitary-Interrenal Axis*

As is the case in most vertebrate species, the hypothalamus and the pituitary regulate the secretion of corticosteroids, with corticotrophin-releasing hormone (CRH) and

adrenocorticotrophic hormone (ACTH) released from their respective regulatory centers (Bonga 1997). In contrast to mammals where corticosteroid production occurs in the adrenal cortex, corticosteroids in fish are synthesized in the interrenal cells located in the head kidney. Unlike the adrenal cortex, interrenal cells do not collectively form a gland, but rather are organized into layers around the posterior cardinal veins whose branches run through the head kidney (Norris and Carr, 2006). Henceforth, the stress axis in fish shall be referred to as the hypothalamic-pituitary-interrenal axis (HPI), which is homologous to the hypothalamic-pituitary-adrenal axis in higher vertebrates. The cortisol secreting cells will be referred to as adrenocortical cells (Hontela and Vijayan, 2009).

When a fish is subjected to a stressor, the hypothalamus releases CRH which stimulates the anterior pituitary to secrete ACTH. Fish have a number of sensory systems that are able to detect stressors including strong light, changes in temperature, pressure, and water-borne pollutant(s), stress-inducing stimuli which will induce an increase in plasma cortisol levels. ACTH is an important regulator of corticosteroid release in fish, although elevated levels of cortisol are not exclusively the result of ACTH stimulation (Bonga, 1997). ACTH acts on the adrenocortical cells, inducing them to begin synthesizing and releasing corticosteroids. The production of corticosteroids is dependent on the steroidogenic acute regulatory (StAR) protein, whose function is to bring cholesterol into the inner mitochondrial membrane (Stocco, 2000). Once inside the inner membrane, cytochrome P450 side-chain cleavage enzyme (P450<sub>scc</sub>) converts cholesterol into pregnenolone, which is a precursor to all steroid hormones. During this process, StAR protein functions as the rate limiting step in the steroidogenic cascade. Cortisol production and secretion is controlled by a negative feedback loop, where excess levels of corticosteroids decrease ACTH and CRH secretion from the pituitary and the hypothalamus (Hontela, 1998).

In fish, cortisol elicits changes in numerous physiological systems. Cortisol targets the intestines, gills, and liver, regulating ion balances and metabolism (Bonga, 1997). Cortisol

secretion also affects immune function, reproductive status, secretion of growth hormone and food intake (Mommsen et al., 1999). One of the primary features of the stress response is the redirection of energy away from growth and reproduction, channeling energy stores towards returning the body to homeostasis, most notably acting on respiration, locomotion, ion balances, and tissue repair (Bonga, 1997). Cortisol triggers gluconeogenesis and lipolysis, which partially explains why chronic stress causes weight loss. Another contributing factor to stress related

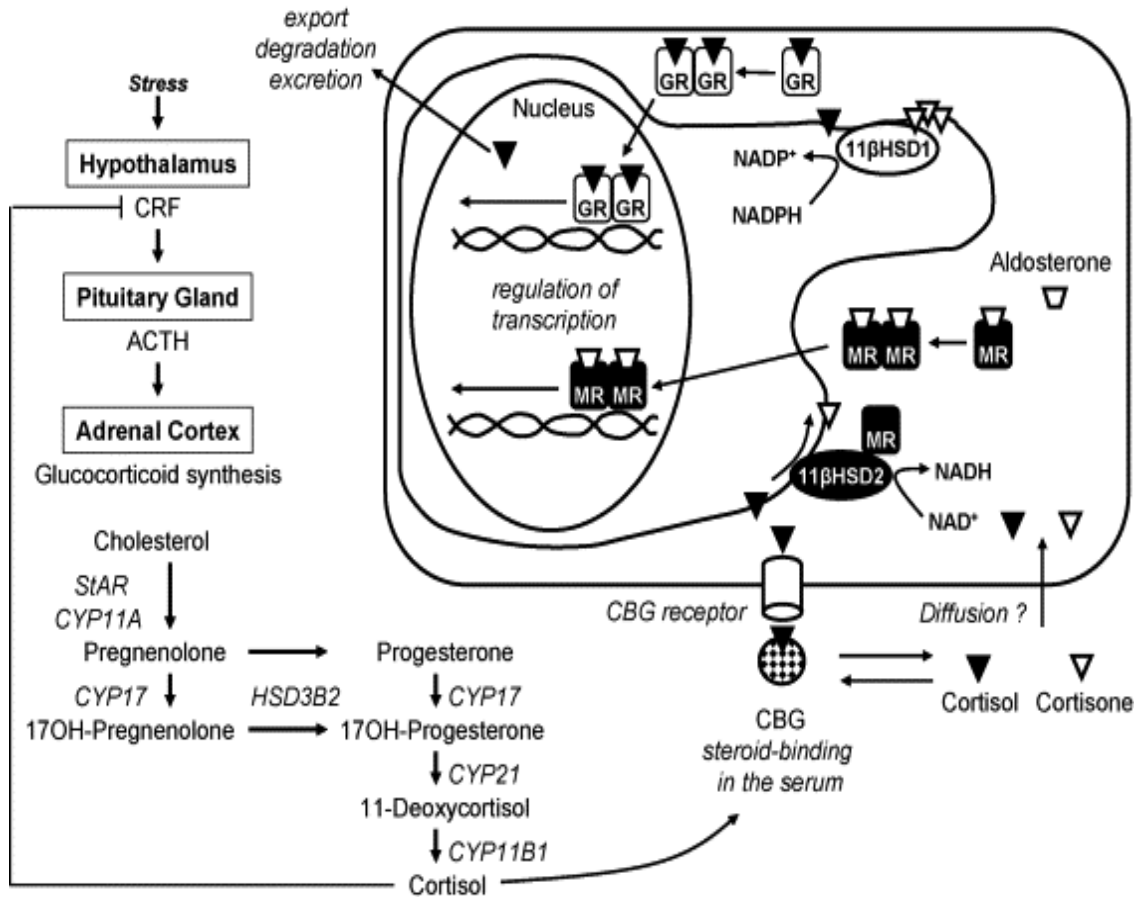


Figure 1.2. Schematic representation of steps involved in glucocorticoid homeostasis (adapted from Odermatt et al., 2006).

weight loss, is the fact that both intense acute and chronic stressors will act as appetite suppressors through the release of CRF, which is known to have anorexigenic effects in the hypothalamic feeding centre of the brain (Bernier and Peter 2001; Bernier 2006).

In salmonid species, cortisol secretion plays an important role in smoltification and spawning migration, and it is only when plasma cortisol levels are perpetually elevated that the response is no longer adaptive (Pickering, 1993). This phenomenon occurs in many different fish species, where acute spikes in cortisol are adaptive and prolonged increases in cortisol have deleterious effects on physiological systems, compromising immune functioning and reproduction. Cortisol is such an important hormone in fish, that if the stress response is blunted by the presence of aquatic pollutants which may disrupt cortisol steroidogenesis, the survivability of fish populations may be jeopardized.

#### *1.7.2 Pollutants and the Stress Response*

Environmental contaminants such as metals, pesticides, and organic chemicals may trigger and/or act to suppress the stress response in fish. Currently, the effect of 2,4-D, TCS, and their by-products, on the HPI axis are unknown. Several researchers have demonstrated that short-term exposures to complex mixtures of chemicals will initially elicit an increase in plasma cortisol and glucose, whereas chronic exposures will eventually exhaust the hypothalamic-pituitary-interrenal axis, as indicated by marked decreases in corticosteroid production (Hontela et al., 1992; Brodeur et al., 1997; Nolan et al., 2003). For example, sub-lethal exposures to mixtures of agricultural chemicals impair the ability of fish to upregulate the production of cortisol in response to an acute stressor (Cericato et al., 2009). Pollutants may also exert their toxic effects on the stress response by damaging adrenocortical cells, thus directly interfering with their ability to produce and secrete corticosteroids (Hontela and Vijayan, 2009). It is also worth noting that during certain periods of the year, fish may be more vulnerable to the effects of toxicants. Winter Stress Syndrome is brought about by a significant decline in the fat reserves of fish, precipitated by both natural and external stressors (Lemly, 1996). During this time, fish are especially susceptible to adverse environmental conditions. The risk posed to fish from chemicals released from wastewater treatment plants, industry, and agriculture may increase significantly. It is likely that global warming and the squaring of the population pyramid will affect the

concentration of pollutants in aquatic environments, as both of these phenomena could very likely increase fish exposure to toxicants.

*In vivo*, high concentrations of serum cortisol are frequently used to indicate exposure to a stressor (Bonga, 1997). This method of assessment is quite reliable as basal levels of cortisol are very low, and it is possible to detect increases in blood cortisol following the exposure to a stressor(s). Endocrine disruption in the stress axis is frequently overlooked by researchers who have the propensity to focus their attention disproportionately on reproductive systems. More research is needed in this area, considering the broad range of regulatory effects that corticosteroids elicit, affecting the ability of fish populations to survive and thrive.

Sentinel species, such as fish, may provide evidence of endocrine disruption in their natural environments (Hontela and Vijayan, 2009). To further investigate the role of environmental contaminants in the disruption of the HPI axis, interrenal cells can be harvested, exposed to the toxicant(s), and then challenged with ACTH *in vitro*. If the ability of these cells to respond to ACTH is blunted or blocked, the stress response in the fish may be impaired. The ACTH challenge test is routinely performed in endocrine toxicology to determine the effect of a toxicant(s) on the HPI axis. An advantage of primary cell cultures is that they tend to retain the characteristics of the tissue from which they were excised, meaning that the *in vitro* results are usually a fairly accurate representation of what would be expected to occur *in vivo*.

Primary cell cultures of fish interrenal cells are an important tool for the ecotoxicological testing of commonly detected water-borne contaminants. This cell bioassay screens for the endocrine disrupting effects of water-borne chemicals, but is also well suited as a diagnostic tool for the determination of the cellular and molecular mechanisms causing the disruption. In the past, scientists have focused the bulk of their attention on the estrogenic potential of endocrine disruptors, through mechanisms involving direct binding to estrogen receptors. The rainbow trout adrenocortical cell bioassay is not a measure of endocrine disruption via specific receptor

binding, but instead provides researchers with a tool for identifying chemicals that impair cortisol biosynthesis, including chemicals acting through indirect mechanisms.

A potentially important non-receptor mediated mechanism of action is the suppression of steroidogenic enzyme activity and/or alterations in key proteins, most notably P450<sub>scc</sub> and StAR protein (Arukwe, 2008). In addition to the adrenal gland, steroids are synthesized in the testis, ovary, brain, placenta, and fat deposits. The rate-limiting step in the synthesis of all steroid hormones is the transfer of cholesterol from the outer to the inner mitochondrial membrane. The commencement of steroidogenesis is marked by the conversion of cholesterol to pregnolone by cytochrome P450<sub>scc</sub>. Due to similarities in steroid hormone synthesis pathways, xenobiotic-induced alterations to the expression of StAR and P450<sub>scc</sub> in adrenocortical cells will likely carry over to other steroid producing tissues.

A number of chemicals induce adrenocortical and steroidogenic toxicity, including pesticides, pharmaceuticals, and heavy metals (Walsh et al., 2000; Hilscherova et al., 2004; Zhao et al., 2005). Considering that the adrenal gland is the most common target for endocrine disruption (Harvey et al, 2007), more research is needed to identify adrenal toxicants and determine the mechanism(s) of action. *In vitro* bioassays based on the ACTH challenge test are excellent screening tools for assessing adrenocortical inhibition, identifying molecular targets, and measuring steroids, enzymes, or gene expression (Harvey et al., 2007).

## **1.8 Hypotheses**

A number of different water-borne chemicals alter steroidogenesis by acting on several endocrine targets, including steroid hormone receptors, cholesterol production, StAR protein, and other steroidogenic enzymes. Based on the structural similarities of TCS, M-TCS, 2,4-D and DCP to known endocrine disruptors, it was hypothesized that the exposure of rainbow trout interrenal cells to these chemicals would alter their ability to produce cortisol in response to ACTH stimulation.

## 1.9 Objectives

The objective of this project was to determine the effect(s) of single toxicants and binary mixtures on the corticosteroid producing cells of the interrenal tissue of rainbow trout. Binary mixtures of chemicals may have additive or synergistic toxicity. The  $EC_{50}$  (Effective concentration of the test toxicant which inhibits 50% of the normal secretory response to ACTH) of the parent compounds was used to determine how the addition of a by-product affected the toxicity of the parent-compound. There is very little information on how the toxicity of the by-products, M-TCS and DCP, compares to that of their parent-compounds, TCS and 2,4-D. This study sought to determine if the by-products were more toxic, less toxic, or equally toxic in relation to their parent compounds.



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## CHAPTER 2. TRICLOSAN: ENVIRONMENTAL EXPOSURE, TOXICITY AND MECHANISMS OF ACTION

*(Invited literature review, Dann and Hontela, 2011 J Applied Toxicol, 31, 285-311)*

### 2.1 Background

Triclosan [(5-chloro-2-(2,4-dichlorophenoxy)phenol: TCS], a halogenated phenol, is a non-ionic, broad spectrum antimicrobial used throughout North America, Europe, and Asia, as an ingredient in disinfectants, soap, detergent, toothpaste, mouthwash, fabric, deodorant, shampoo, and plastic additives, in addition to innumerable other personal care, veterinary, industrial and household products. TCS is effective against many types of bacteria and certain types of fungi, preventing bacterial propagation and/or eventually resulting in cell death. It permeates the bacterial cell wall and targets multiple cytoplasmic and membrane sites, including RNA synthesis and the production of macromolecules (Russell, 2004). TCS also blocks synthesis of fatty acids through inhibition of enoyl reductase, but has no effect on bacterial spores (McMurray et al., 1998; Levy et al., 1999; Russell, 2004). TCS may be classified as a halogenated aromatic hydrocarbon, containing phenol, diphenyl ether, and polychlorinated biphenyl functional groups (Ahn et al., 2008). The chemical structure of TCS (Fig. 1), a halogenated biphenyl ether, is similar to polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), bisphenol A, dioxins, and thyroid hormones (Veldhoen et al., 2006; Cabana et al., 2007; Crofton et al., 2007; Allmyr et al., 2008), molecules with two aromatic rings.

TCS was invented over 40 years ago and has been used increasingly over the past 25 years (Jones et al., 2000; Russell, 2004). In the period from 1992 to 1999, a majority of the 700 antibacterial products on the market contained TCS as an active ingredient (Schweizer, 2001). TCS is the generic name for the chemical, with brand names including Irgasan DP300, Aquasept, Sapoderm, and Ster-Zac. Fibres and other materials that have TCS incorporated into them may be referred to as Ultra-Fresh, Amicor, Microban, Monolith, Bactonix, and Sanitized (Adolfsson-Erici et al., 2002). The antimicrobial has the capability to migrate from treated surfaces into



foodstuffs. Notwithstanding, the addition of TCS to food coverings and surfaces that are in contact with food during processing is currently being considered (Canosa et al., 2008). However, as of March 2010, TCS was removed from the EU list of provisional additives for use in plastic food-contact materials.

Unlike some other organochlorine compounds, TCS use is not highly regulated, as the antimicrobial has a low acute toxicity and is generally accepted as well tolerated and safe (Jones et al., 2000; Rodericks et al., 2010). Concentrations of TCS in personal care products are typically in the range of 0.1-0.3% of product weight (Sabaliunas et al., 2003), with significant amounts of the antimicrobial entering wastewater treatment facilities (Table 2.1). The prevalence of TCS in waterways is likely to increase as consumer demand for antimicrobial products is anticipated to grow. TCS is being increasingly scrutinized after concerns emerged that the product might be harmful to human health and the environment. TCS has been detected in surface water, sediment, biosolids, soils, aquatic species, and humans (Chu and Metcalfe, 2007; Chalew and Halden, 2009; Reiss et al., 2009). Potential health issues surrounding the use of TCS include antibiotic resistance, skin irritations, endocrine disruption, increasing rates of allergies, and the formation of carcinogenic by-products (Schweizer, 2001; Adolfsson-Erici et al., 2002; Latch et al., 2003), yet a recent review by Rodericks et al. (2010) concluded that exposure to TCS in consumer products is not expected to cause adverse health effects in children or adults who use the products as intended.

Despite the widespread use of TCS, few independently published studies have investigated the emerging health concerns surrounding the use of this antimicrobial and the environmental impact it may have. Previous reviews of TCS focused primarily on its toxicity in laboratory animals and humans, its fate in the environment, or its link to antibiotic cross-resistance. The objective of this review is to provide a comprehensive review of the literature on TCS, its occurrence in aquatic and terrestrial environments, exposure levels in humans and wildlife, including aquatic species, its toxicity and endocrine disrupting potential.

## 2. 2 Identity, physical and chemical properties, manufacture, and use

### 2.2.1 Identity, physical, and chemical properties

Triclosan (CAS registration number of 3380-34-5), is a diphenyl ether and may be referred to as 5-chloro-2-(2,4-dichlorophenoxy)phenol or 2,4,4'-trichloro-2'-hydroxydiphenyl ether. The molecular formula for TCS is  $C_{12}H_7Cl_3O_2$  and the chemical has a molecular weight of 289.55. Most commercially obtained grades of TCS are over 99% pure and are available in the solid form as a white to off-white crystalline powder with a barely detectable aromatic odour. TCS is a stable compound with a boiling point between 280-290°C, and a melting point between 54-57°C. The thermal stability of TCS is why certain manufacturers have chosen the antimicrobial for the incorporation into plastics and fibers. The octanol/water partition coefficient ( $\log K_{ow}$ ) of TCS is 4.76; it is not readily soluble in water (10 mg l<sup>-1</sup> at 20°C), although solubility increases as the pH becomes more alkaline. TCS is however, easily dissolved in a wide array of organic solvents (Bhargava and Leonard, 1996).

In aquatic ecosystems, the majority of TCS exists in the ionized form (Orvos et al., 2002) and it is primarily the un-ionized form that is responsible for the majority of TCS's toxic effects. The half-life of TCS in surface water is approximately 41 min, with most of the parent compound converting to 2,4-dichlorophenol, although degradation rates vary considerably across aquatic ecosystems (Reiss et al., 2002; Lyndall et al., 2010).

### 2.2.2 Manufacture and use

During the synthesis of TCS, a chlorinated phenoxyphenol, the potential for contamination with toxic impurities exists. Beck et al. (1989) reported trace amounts of lower chlorinated dibenzodioxins and furans in Irgasan DP300, but not in excess of the  $\mu\text{g kg}^{-1}$  range, leading to the conclusion that presence of these compounds in TCS is of little concern. Low levels of dioxins and dibenzofurans may be present as unwanted by-products, depending on the quality of the initial materials used to synthesize TCS, as well as manufacturing conditions such as temperature and pressure (Ni et al., 2005). The U.S. EPA (1994) considers that TCS may be

potentially contaminated with dioxins, with the EU, Canada and the United States having taken initiatives to set standards for maximum permissible levels of impurities in this compound.

Between 1992 and 1999, over 700 antibacterial products, the majority of which contained TCS, entered the consumer market. Personal care products are the most common form of exposure to the antimicrobial, typically at concentrations of 0.1% up to 0.3%, levels which are regulated by the European Community Cosmetic Directive or the US Food and Drug Agency (USFDA) in Europe and the United States, respectively (Sabaliunas et al., 2003; Rodricks et al., 2010). In Sweden, 25% of toothpaste brands contain TCS which translates into two tons of TCS consumption per year. Soaps, deodorants, and other personal care products account for another 300 kg of the chemical in Sweden alone (Adolfsson-Erici et al., 2002). On the global front, the production of TCS has now exceeded 1500 tons per year, with Europe being responsible for 350 tons of total production (Singer et al., 2002). As public concern over the transmission of disease is heightened, the use of antimicrobials is anticipated to increase. TCS will continue to be an environmental pollutant that warrants monitoring, especially since its transformation products are not yet fully understood.

## **2.3 Environmental exposure**

### *2.3.1 Occurrence in the aquatic environment*

#### 2.3.1.1 Triclosan

The antimicrobial TCS is commonly detected in aquatic ecosystems (Table 2.1; Capdevielle et al., 2008; Chalew and Halden, 2009; Lyndall et al., 2010). The majority (96%) of consumer products containing TCS are eventually rinsed down the drain (Reiss et al., 2002) and discharged with wastewater effluent. Although wastewater treatment plants (WWTP) are generally highly effective in removing TCS, a small percentage of the antimicrobial is usually discharged with effluent into receiving waters, usually a river system (Morrall et al., 2004; Nakada et al., 2008). The efficiency of TCS removal can be highly variable, with elimination

rates ranging from complete removal to 100% ineffective (Kanda et al., 2003; Heidler and Halden, 2007).

The variability in removal rates for TCS is due in part to different treatment processes, as the antimicrobial is readily degraded in aerobic conditions but not under anaerobic conditions (McAvoy et al., 2002). Field measurements from a Swiss WWTP have detailed the elimination process of TCS: 79% was biologically degraded, 15% was sorbed to sludge, and 6% left the plant in the final effluent at a concentration of 42 ng l<sup>-1</sup> (Singer et al., 2002; Table 2.1). These results are consistent with tests conducted at several WWTPs in Germany, where 4 - 10% of TCS remained dissolved in out-flowing water (Bester, 2003). Generally, WWTP influent concentrations of the antimicrobial range from 1.86-26.8 µg l<sup>-1</sup>, with effluent concentrations ranging from 0.027 to 2.7 µg l<sup>-1</sup> (Morrall et al., 2004; Chalew and Halden, 2009; Nakada et al., 2010). In the period from 1999 to 2000, the U.S. Geological Survey detected TCS in 57.6% of streams and rivers sampled, at concentrations ranging from below the detection limit up to 2.3 µg l<sup>-1</sup> (Kolpin et al., 2002). In addition to the incomplete removal from WWTP effluent, the antimicrobial exhibits a tendency to accumulate and persist in biosolids; it is estimated that up to 50% of TCS in WWTP influent will remain in biosolids, in WWTPs which utilize activated sludge treatments in combination with anaerobic biosolid digestion (Heidler and Halden, 2006; Chalew and Halden, 2009; Lozano et al., 2010). The TCS removal capacities of various sorbents, including activated charcoal and kaolinite, and the effects of pH, ionic strength and humic acid on the sorptive interactions have been investigated (Behera et al., 2010). Organic matter content was a major factor controlling the sorption of TCS. The occurrence of TCS and other organic contaminants has been reported in Canadian municipal sewage sludge and biosolids samples (Lee and Peart, 2002; Chu and Metcalfe, 2007; Mackay and Barnhouse, 2010; Table 2.1). Thus the two main sources of TCS release into the environment are: 1. discharge of WWTP effluent into receiving waters, and 2. land application of biosolids containing residues of the antimicrobial.

A multitude of factors influence TCS concentrations in aquatic systems, including the TCS load in effluent, physical and chemical properties of TCS, characteristics of the aquatic ecosystem (pH, sediment density and organic matter content, water flow and velocity, depth), and even season and intensity of sunlight (Reiss et al., 2002; Trixier et al., 2002; Lyndall et al., 2010). TCS has been measured not only in surface waters, but also in freshwater and estuarine sediment, at concentrations of 800 to 53,000  $\mu\text{g kg}^{-1}$  (Miller et al., 2008; Chalew and Halden, 2009; Table 2.1). Monitoring TCS concentrations in surface water is important, as the antimicrobial has demonstrated a propensity for bioaccumulation in aquatic species (Balmer et al., 2004) and can persist in aquatic ecosystems for extended periods of time. The antimicrobial has been measured in 30 year old sediment from lake Greifensee in Switzerland (Singer et al., 2002). This study provided evidence of the persistence of TCS in sediment and detailed the pattern of use of TCS. TCS concentrations in sediment increased between the early 1960's until the mid-1970's, reflecting steadily increasing patterns of use, then a reverse in this trend was observed from the mid-1970's until the early 1980s, when a new process of wastewater treatment was introduced into most WWTPs. Increases in TCS concentrations occurred again from the early 1980's until the present time. Similar depth-time profiles for TCS spanning last 40 years were reported by Miller et al. (2008) for estuarine sediments in the US. The environmental persistence of TCS in sediment is indicative of the antimicrobial's potential to partition into sediment and resist degradation processes under anaerobic conditions. Buth et al. (2009) chronicled the historical pattern of dioxin photoproducts of TCS and its chlorinated derivatives in sediment cores from the Mississippi river. Between 1963-2008, TCS levels markedly increased, corresponding to increases in the concentration of several chlorinated derivatives of TCS (CTDs), including dichlorodibenzo-*p*-dioxin (2,8-DCDD), a direct transformation product of the photolysis of TCS. A further source of TCS derived dioxins comes from the solar irradiation of CTDs, leading to the formation of higher level chlorinated dioxins.

**Table 2.1.** Concentrations of Triclosan (TCS) in the aquatic environment

Medium	Sample Description	Location	Concentration of TCS	Reference
Surface Water	Natural streams/rivers	United States	ND <sup>a</sup> - 2.3 $\mu\text{g l}^{-1}$	Kolpin et al. 2002; Morrall et al. 2004
		Switzerland	ND-0.074 $\mu\text{g l}^{-1}$	Lindstrom et al. 2002
		Germany	ND-0.01 $\mu\text{g l}^{-1}$	Bester, 2005
		Sweden	ND	Bendz et al. 2005
		Australia	0.075 $\mu\text{g l}^{-1}$	Ying and Kookana, 2007
		Japan	<0.0006-0.059 $\mu\text{g l}^{-1}$	Nakada et al. 2008
	Streams with inputs of raw wastewater	Switzerland	0.011-0.098 $\mu\text{g l}^{-1}$	Singer et al. 2002
		United States	1.6 $\mu\text{g l}^{-1}$	Halden and Paull, 2005
	Estuarine waters	United States	0.0075 $\mu\text{g l}^{-1}$	Fair et al. 2009
Sediment	Freshwater	Switzerland	53 $\mu\text{g kg}^{-1}$	Singer et al. 2002
		Spain	ND-35.7 $\mu\text{g kg}^{-1}$	Morales et al. 2005
	Estuarine	United States	ND-800 $\mu\text{g kg}^{-1}$	Miller et al. 2008
	Marine	Spain	0.27-130.7 $\mu\text{g kg}^{-1}$	Aguëra et al. 2003

Sewage Sludge	Activated Sludge	Waste	United States	0.5-15.6 $\mu\text{g g}^{-1}$	McAvoy et al. 2002
			Spain	0.4-5.4 $\mu\text{g g}^{-1}$	Morales et al. 2005
			Germany	1.2 $\mu\text{g g}^{-1}$	Bester, 2003
			Canada	0.62-1.45 $\mu\text{g g}^{-1}$	Chu and Metcalfe, 2007
	Biosolids		Australia	90-16 790 $\mu\text{g kg}^{-1}$	Ying and Kookana, 2007
			United States	10 500-30 000 $\mu\text{g kg}^{-1}$	Kinney et al. 2008; Heidler et al. 2007
			Spain	1508 $\mu\text{g kg}^{-1}$	Morales et al. 2005
			Canada	680-12 500 $\mu\text{g kg}^{-1}$	Lee and Peart, 2002; Chu and Metcalfe, 2007
WWTP Influent	In-Flowing Water	United States	2.70-26.80 $\mu\text{g l}^{-1}$	McAvoy et al. 2002; Halden and Paull, 2005; Heidler and Halden. 2007; Waltman et al. 2006; Fair et al. 2009	
		Canada	0.01-4.01 $\mu\text{g l}^{-1}$	Lishman et al. 2006	
		Germany	1.2 $\mu\text{g l}^{-1}$	Bester, 2003	
		Sweden	0.38 $\mu\text{g l}^{-1}$	Bendz et al. 2005	
		Japan	2.7-11.9 $\mu\text{g l}^{-1}$	Nakada et al. 2010	

WWTP Effluent	Treated Water			
		Switzerland	0.042-0.213 $\mu\text{g l}^{-1}$	Singer et al. 2002
		Germany	0.01-0.6 $\mu\text{g l}^{-1}$	Bester, 2003; 2005
		Canada	0.01-0.324 $\mu\text{g l}^{-1}$	Lishman et al. 2006
		United States	0.03-2.7 $\mu\text{g l}^{-1}$	McAvoy et al. 2002; Waltman et al. 2006; Heidler and Halden, 2007; Halden and Paull, 2005; Fair et al. 2009
		United Kingdom	0.34-3.1 $\mu\text{g l}^{-1}$	Kanda et al. 2003; Sabaliunas et al. 2003
		Australia	0.023-0.434 $\mu\text{g l}^{-1}$	Ying and Kookana, 2007
		Sweden	0.16 $\mu\text{g l}^{-1}$	Bendz et al. 2005
		Japan	0.26-0.27 $\mu\text{g l}^{-1}$	Nakada et al. 2010

<sup>a</sup> ND= Not detectable



### 2.3.1.1 Degradation products of TCS

Methyltriclosan (MTCS)- During the wastewater treatment process, TCS is transformed by biological methylation into methyltriclosan (MTCS; 5-chloro-2-(2,4 dichlorophenoxy)anisole; CAS No. 4640-01-1) (Boehmer et al., 2004; Bester, 2005), a more lipophilic compound ( $K_{ow}$  5.2), which is then released into receiving waters. The presence of MTCS in fish has in fact been proposed for use as marker of exposure to WWTP effluent, specifically to lipophilic WWTP contaminants (Balmer et al., 2004). The lipophilicity of MTCS and its resistance to biodegradation processes and photolysis (Lindström et al., 2002) means this metabolite will exhibit a higher degree of environmental persistence than its parent compound.

Dioxins- Over the last decade there has been increasing concern regarding the degradation products of TCS, most notably dioxins, and consequently, the transformation of TCS during manufacturing, incineration, and in the aquatic environment. The photolysis of TCS constitutes the principal removal pathway of the antimicrobial in the aquatic environment, with some studies having documented the formation of 2,8-dichlorodibenzodioxin (DCDD) and other dioxin derivatives during the photodegradation of TCS in aqueous solutions (Latch et al., 2003; Mezcua et al., 2004; Lores et al., 2005; Sanchez-Prado et al., 2006; Aranami and Readman, 2007).

There is evidence that the pH of aqueous solutions spiked with TCS influences the formation of dioxin by-products. Latch et al. (2003) reported that 1-12% of TCS is converted to DCDD in aqueous solutions buffered at a pH 8 or higher. Considering the pKa of TCS is 7.9, it is probable that the dissociated form of TCS is the photoreactive species, potentially explaining why DCDD was not observed in experiments using methanol solutions spiked with TCS (Latch et al., 2003). From this study, it is apparent that in sunlight irradiated waters, the conversion of TCS into dioxin by-products is dependent on both the pH and the irradiation wavelength. The findings of Latch et al. (2003) were confirmed by Mezcua et al. (2004) who were the first to investigate the photodegradation of TCS to dioxins in wastewater samples. The study indicated that 2,7/2,8-dibenzodichloro-*p*-dioxin is indeed a by-product of the photolysis of TCS, in both water and

wastewater samples spiked with  $8 \mu\text{g ml}^{-1}$  of the antimicrobial. The degree of photolytic conversion was dependent upon pH and the organic matter content in the sample. Sanchez-Prado et al. (2006) were the first to use a solar simulator photoreactor, in conjunction with actual contaminated wastewater samples, identifying the formation of 2,8-DCDD and a possible DCDD isomer or dichlorohydroxydibenzofuran independently of sample pH. Aranami and Readman (2007) irradiated freshwater and seawater samples with a low intensity artificial white light source for a 12 day period. Similar to previous studies, the photodegradation of TCS produced DCDD, in both freshwater and seawater samples after 3 days of irradiation.

The photochemical conversion of TCS in natural water samples, specifically Mississippi river and Lake Josephine waters, was investigated by Buth et al. (2009). The photolysis of the antimicrobial was dependent on speciation, with the phenolate form of TCS being degraded 44-586 times faster than the phenol form. The conversion of chlorinated TCS derivatives into dioxins was substantiated in natural and buffered pure water, with yields of 0.5 to 2.5%, respectively. The majority of TCS's photolytic transformation products and their kinetics, along with the environmental factors influencing their degradation, have yet to be identified. (Aranami and Readman, 2007). Of great importance in quantifying the level of risk to both aquatic environments and humans, is determining to what extent and under which environmental conditions the conversion of TCS into toxic by-products occurs.

Chlorophenols - Dioxins are not the only toxic transformation product of TCS that warrants further study. The photochemical transformation of TCS has also been shown to produce 2,4-dichlorophenol and 2,4,6-trichlorophenol, chemicals which the US EPA has flagged as priority pollutants. The generation of chlorophenols from TCS was originally demonstrated by Kanetoshi et al. (1987) however, the study used high concentrations of chlorine and TCS, calling into question the environmental relevance of the findings. Later studies validated the finding that chlorophenols are transformation products of TCS, even in the presence of low levels of chlorine or chloramines (Rule et al., 2005; Canosa et al., 2005; Greyschock and Vikesland, 2006). TCS

reacted with free chlorine under drinking water conditions and 2,4-dichlorophenol was formed via the ether cleavage of TCS, which then underwent electrophilic substitution to form 2,4,6-trichlorophenol. Consistent with other studies, based on the effect of pH on the formation of TCS by-products, Rule et al. (2005) concluded that it was primarily the ionized phenolate form of TCS that reacts with hypochlorous acid. Canosa et al. (2005) tested low concentrations of both TCS ( $\text{ng ml}^{-1}$ ) and chlorine ( $\text{mg l}^{-1}$  and less), and consistently detected 2,4-dichlorophenol and 2,4,6-trichlorophenol in all of the samples analyzed. Even though the molar yields of TCS conversion were  $<10\%$ , these findings are significant, as it has been demonstrated that these two phenolic by-products are relatively stable over time and potentially toxic (Canosa et al., 2005). The formation of chlorophenols from the degradation of TCS has been confirmed by others (Sanchez-Prado et al., 2006; Latch et al., 2005; Fiss et al., 2007).

Chloroform -There is evidence that, like other phenols, TCS in water or in various consumer products will react with free chlorine or chloramine to produce chloroform and other chlorinated products over a range of pHs (Rule et al., 2005; Greyslock and Vikesland, 2006; Fiss et al., 2007). Rule et al. (2005) also assessed the propensity of a dish soap containing TCS to form chloroform when added to chlorinated water. After 5 min,  $15 \mu\text{g l}^{-1}$  of chloroform was produced, with chloroform levels attaining  $49 \mu\text{g l}^{-1}$  after 120 min. Based on the results of this study, while it is unlikely that significant amounts of chloroform are generated from TCS in surface waters, chloroform may be formed during the daily use of household products containing the antimicrobial. The conversion of TCS to chlorinated derivatives is also dependent on temperature, with higher temperatures resulting in increased chloroform yields (Fiss et al., 2007). An exposure model completed by the authors indicated that, under certain conditions, the amount of chloroform produced could be significant, and where chloroform formation is inconsequential, other chlorinated by-products are produced, which may place consumers at an increased risk for adverse health effects.

## 2.4 Exposure to Triclosan and its degradation products in aquatic organisms

### 2.4.1 Algae and Invertebrates

The incomplete removal of TCS during the wastewater treatment process leads to the continual exposure of aquatic biota in receiving waters, and the accumulation of the antimicrobial and its degradation products in tissues of aquatic organisms (Table 2.2). Algae, a primary food source for many aquatic species, constitute an important pathway for the accumulation of lipophilic water-borne contaminants, such as TCS (Capdeville et al., 2008). Coogan et al. (2007) sampled the filamentous algae (*Cladophora* spp) in a receiving stream for the city of Denton (Texas) for TCS and MTCS, measuring 100 - 150  $\mu\text{g kg}^{-1}$  and 50-89  $\mu\text{g kg}^{-1}$ , respectively. From these measurements, bioaccumulation factors of 1600 and 1100 were estimated for the parent compound and its methylated by-product. The bioaccumulation potential of TCS and MTCS was also determined in freshwater snails (*Helisoma trivolvis*) and again in algae (*Cladophora* spp.), using isotope dilution GC-MS (Coogan and La Point, 2008). Bioaccumulation factors for snail tissue were 500 and 1200 for TCS and MTCS, respectively. The algal bioaccumulation factor was also high, 1,400 and 1,200, respectively. The occurrence and formation of TCS metabolites was also investigated in estuarine systems. In a study by DeLorenzo et al. (2008), adult grass shrimp (*Palaemonetes pugio*) were exposed to 100  $\mu\text{g l}^{-1}$  of TCS and even though TCS was not measured, they were found to accumulate MTCS after a 14-day exposure period. This finding provides evidence for both the conversion of TCS to MTCS in seawater, and of the bioaccumulation potential of the metabolite in aquatic organisms. Yet, even though MTCS is resistant to biodegradation processes and has demonstrated the ability to persist in the environment for longer periods of time than the parent compound, it has received considerably less attention in the literature. As snails and other aquatic invertebrates depend on algae as a source of nutrients, and considering the ubiquity of TCS in the aquatic environment, it is probable that grazed algal compartments will contain TCS and MTCS, potentially making these compounds available to higher aquatic organisms.

#### 2.4.2 Fish

In addition to invertebrates, TCS and its transformation products have been detected in higher level aquatic organisms, most notably fish (Table 2.2). Miyazaki et al. (1984) was the first to report the presence of MTCS in aquatic biota. Fish and shellfish were collected from the Tama River and Tokyo Bay, and MCTS was identified by GC/MS in all of the freshwater fish samples (1-38  $\mu\text{g kg}^{-1}$  whole body) and 3 of the 4 shellfish samples (3 - 20  $\mu\text{g kg}^{-1}$ , Table 2.2). A well cited study by Adolfsson-Erici et al. (2002), measured TCS levels in rainbow trout (*Oncorhynchus mykiss*) caged in the receiving waters of a WWTP in Sweden, in wild fish living downstream from the plant, and in rainbow trout exposed to treated water in tanks. Bile fluid from the fish contained TCS at concentrations ranging from  $<0.01 - 0.08 \text{ mg kg}^{-1}$  fresh weight in controls and fish sampled at reference sites, and 0.44 - 120  $\text{mg kg}^{-1}$  in fish exposed to sewage water. Houtman et al. (2004) also used GC/MS to identify a multitude of xenobiotic compounds, including TCS, in the bile of male breams (*Abramis brama*) living in Dutch surface waters. TCS was detected in two of the three locations sampled, at relatively high concentrations of 14  $\mu\text{g ml}^{-1}$  and 80  $\mu\text{g ml}^{-1}$  of bile. The results of these two studies provide evidence for the accumulation of TCS in the bile of fish. Other European studies have reported TCS and its derivatives in fish tissues. Buser et al. (2006) analyzed levels of MTCS in juvenile (1-2 year old) brown trout (*Salmo trutta fario*) from rivers in Northern Switzerland receiving effluent from WWTPs. Concentrations of MTCS in fish were reported between 130-2100  $\text{ng g}^{-1}$  of lipid weight. Balmer et al. (2004) detected MTCS in lake fish in the range of 4-370  $\text{ng g}^{-1}$ , lower levels compared to those previously measured in fish samples from rivers. This difference is to be expected as concentrations of MTCS should typically be higher in river systems that receive inputs from WWTPs. A large monitoring study on TCS and MTCS was conducted by Boehmer et al. (2004) using fish tissues from the German Environmental Specimen Bank. Samples of muscle tissue from breams (*Abramis brama*) from the period of 1994-2003 were analyzed for TCS and MTCS. While TCS was only detected in a small number of samples, MTCS was present in all of the

muscle samples analyzed. A pattern of increasing MTCS concentrations was observed in bream muscle tissue from the mid 1990's until after 2000, with levels of MTCS increasing from 10 ng g<sup>-1</sup> to 14-26 ng g<sup>-1</sup> of wet weight. TCS concentrations ranged from below the limit of quantification up to 3.4 ng g<sup>-1</sup>. From their retrospective monitoring data, the authors of the study concluded that MTCS is a persistent pollutant with the potential to accumulate in the muscle tissue of fish.

**Table 2.2.** Concentrations of Triclosan (TCS) in aquatic organisms

Organisms	Type of Sample	Site Description	TCS ( $\mu\text{g kg}^{-1}$ )	Reference	
<b>Algae and Invertebrates</b>					
Filamentous ( <i>Cladophora spp</i> )	algae	Whole organism	Receiving stream for the city of Denton (TX,USA) WWTP	100-150	Coogan et al. 2007
		Whole organism		50-400	Coogan and La Point, 2008
Freshwater ( <i>Helisoma trivolvis</i> )	snails	Muscle		50-300	Coogan and La Point, 2008
<b>Vertebrates</b>					
Rainbow trout ( <i>Oncorhynchus mykiss</i> )		Bile	Upstream from WWTP, Sweden (caged); Downstream 2km from WWTP (caged)	710 17000	Adolfsson-Erici et al. 2002
Breems, ( <i>Abramis brama</i> )	male	Bile	River sites (Netherlands)	14000-80000	Houtman et al. 2004
		Muscle	River sites (Germany)	0.25-3.4	Boehmer et al. 2004
Pelagic fish		Plasma	Detroit river (USA)	0.75-10	Valters et al. 2005
Atlantic Dolphins ( <i>Tursiops truncates</i> )	Bottlenose	Plasma	Estuary, South Carolina Estuary, Florida	0.12-0.27 0.025-0.11	Fair et al. 2009
Killer ( <i>Orcinus orca</i> )	Whale	Plasma	Vancouver Aquarium Marine Science Centre	9.0	Bennett et al. 2009

To date, only a few North American studies have monitored TCS and MTCS levels in freshwater fish (Table 2.2). Valters et al. (2005) detected TCS in the plasma samples of 13 species of fish sampled from the Detroit River, in the range of 750 to >10 000 pg g<sup>-1</sup> of wet weight. MTCS was also detected in the plasma samples, albeit in much lower concentrations, ranging from 0.4-13.4 pg g<sup>-1</sup> of wet weight. Based on these plasma samples, the authors estimated the body burden of TCS to be 2 to 67 ng. Leiker et al. (2009) identified MTCS in male common carp (*Cyprinus carpio*) from the Las Vegas Bay and in the Las Vegas Wash, Nevada; MTCS was detected in all carp sampled (n=29), with a mean concentration of 520 of 596 µg kg<sup>-1</sup> per wet weight basis. The concentrations of MTCS detected in this study were much higher than those documented in previous studies, with the authors indicating that this might be due to the sediment foraging behaviour of carp, which exposes them to higher levels of lipophilic water-borne chemicals than other fish species. TCS and its metabolites have been detected in sediments, both freshwater and marine (Agüera et al., 2003; Miller et al., 2008; Chalew and Halden, 2009). A national pilot study in the United States surveyed the presence of pharmaceuticals and personal care products, including TCS, in fish sampled from five effluent-dominated rivers receiving discharge from WWTPs in large urban centers and a reference river (Ramirez et al., 2009). Although several products, including carbamazepine and norfluoxetine were detected at ng g<sup>-1</sup> concentrations in fish tissues, GC/MS analysis revealed only trace amounts of TCS in fillets.

#### 2.4.3 Marine mammals

Fair et al. (2009) characterized the occurrence of TCS in the plasma of wild Atlantic bottlenose dolphins (*Tursiops truncatus*), a top level predator, and then correlated biological levels with environmental concentrations. This study was the first to document the bioaccumulation of TCS in a marine mammal. Plasma samples were collected from the dolphins in Charleston, South Carolina and Indian River Lagoon, Florida, two southeast US estuarine sites. TCS measured in estuarine water samples ranged from 4.9 -13.7 ng l<sup>-1</sup>, averaging 7.5 ng l<sup>-1</sup>. Plasma concentrations ranged from 0.12 - 0.27 ng g<sup>-1</sup> and 0.025-0.11 ng g<sup>-1</sup> wet weight, at the two



estuaries respectively. Subsequently, TCS has also been detected at a concentration of  $9.0 \text{ ng g}^{-1}$  of wet weight in the plasma of a captive killer whale (*Orcinus orca*) fed a diet of herring harvested from the coast of British Columbia (Bennett et al., 2009). These studies further highlight the need to monitor TCS and assess its effects in wild species.

## **2.5 Occurrence of TCS and its derivatives in the terrestrial environment**

Although TCS is considered to be primarily a water-borne contaminant, the antimicrobial can, and does, enter the terrestrial environment during the application of sewage sludge to agricultural and/or industrial land (Lozano et al., 2010; Fuchsman et al., 2010). Activated sludge concentrations of TCS are typically measured between  $580\text{-}14,700 \text{ }\mu\text{g kg}^{-1}$  of dry weight, whereas concentrations in biosolids have been documented in the range of  $90\text{-}32,900 \text{ }\mu\text{g kg}^{-1}$  (Chalew and Halden, 2009; Lozano et al., 2010). Studies across three continents examined TCS levels in sewage WWTP sludge and reported similar concentrations of the antimicrobial, with a median concentration of  $5000 \text{ }\mu\text{g kg}^{-1}$  of dry weight (Reiss et al., 2009). In WWTPs that use activated sludge treatment in combination with anaerobic biosolid digestion,  $50\pm 19$  of the influent mass of TCS will accumulate and persist in sewage sludge (Chalew and Halden, 2009). Although it is clear that the amendment of agricultural lands with biosolids produced from WWTPs represents a significant pathway for the release of TCS into the terrestrial environment, the environmental impact of land amendment practices that use biosolids from WWTPs has not been assessed.

To predict the effects of TCS in biosolids on the terrestrial environment, it is necessary to understand its fate in soil. Ying et al. (2007) investigated the biological degradation of TCS in soil under aerobic and anaerobic conditions. Quantitative structure-activity relationship analyses confirmed findings from previous studies, demonstrating TCS's propensity to sequester in soil and sediment. Laboratory experiments under aerobic conditions showed that TCS had a half-life of 18 days. However, the antimicrobial persisted in anaerobic soil for the entire duration of the 70 day experiment. These findings agree with McAvoy et al. (2002) who reported that the bulk of TCS in WWTPs was removed during aerobic sludge digestion, with anaerobic sludge digestion

accounting for a very small portion of TCS removal. Thus in both terrestrial and aquatic environments, the biodegradation of TCS occurs primarily under aerobic conditions as the antimicrobial is resistant to anaerobic degradation. Due to TCS's lipophilic nature, the antimicrobial partitions into sediment and soil, but its transport potential from biosolids into surface runoff has been characterized as low (Sabourin et al., 2009).

Although TCS may not be physically mobile between soil compartments, other processes may transfer TCS from soil to biota. Kinney et al. (2008) assessed the potential for organic biosolid- or manure-derived soil contaminants in amended agriculture land to accumulate in biota. Tissue concentrations of TCS in earthworms inhabiting the amended soil reached 2610  $\mu\text{g kg}^{-1}$ , translating into a bioaccumulation factor of 27. Based on the findings of this study, the predation of earthworms by birds and other animals could result in the transfer of TCS up the food chain, although this has not yet been documented.

## **2.6 Human exposure and levels**

The presence of TCS in human tissues has been documented by a number of studies from populations in Europe, USA, and Australia, which would be expected considering the number of personal care products containing the antimicrobial and the ability of TCS to be absorbed dermally (Queckenberg et al., 2010).

### *2.6.1 Urine*

Measuring the levels of environmental chemicals, such as TCS, in urine represents an important biomonitoring tool for exposure assessment, especially considering TCS and its metabolites are excreted primarily in urine (Queckenberg et al., 2010). One of the earlier characterizations of the baseline excretion of TCS in urine, along with plasma levels, was published by Sandborgh-Englund et al. (2006). Five male and five female subjects with a median age of 28 years were exposed to a single oral dose of TCS, and urine and blood samples were collected before and up to 8 days after exposure. The baseline excretion of TCS was in the range of 0.1 to 743  $\mu\text{g d}^{-1}$ , and although maximal plasma levels were reached within 1 - 3 h in all

subjects, plasma levels varied considerably, ranging between 0.1 to 8.1  $\mu\text{g l}^{-1}$ . Neither baseline urinary excretion of TCS nor plasma levels were correlated with the use of TCS containing personal care products. The authors provided three possible explanations for these unexpected results: 1. the monitoring of personal hygiene products was not exhaustive, 2. labelling of product contents was not complete, 3. other sources of exposure explain the variable baseline levels. Indeed, other sources of TCS exposure may include sportswear items, shoes, socks, and impregnated household items (Adolfsson-Erici et al., 2002). The full range of consumer, industrial, and pharmaceutical products that contain TCS needs to be included in future exposure assessment studies in humans.

To further assess the exposure to TCS in a representative sample of the U.S. population, the National Health and Nutrition Examination Survey (NHANES) collected 2,517 urine samples and detected TCS in 74.6% of the samples at concentrations of 2.4-3,790  $\mu\text{g l}^{-1}$  (Calafat et al., 2008). TCS concentrations varied with age and socio-economic status, but not race/ethnicity or sex. Concentrations were the highest amongst people in their 30's and those with higher household incomes. The high frequency of detection resulting from this study is not surprising, since a significant proportion of personal care products on the market today contain TCS, with personal care products considered the primary route of exposure.

As with other toxicants, including potential endocrine disruptors, childhood exposures are a concern, as it is uncertain how these chemicals may alter growth and development processes. In light of the high potential for exposure to TCS, a better understanding of TCS exposure and levels in children is urgently needed. A pilot study by Wolff et al. (2007) collected urine samples from 90 girls between the ages of 6 and 8. Sampling was conducted in a manner that ensured that participants represented four racial groups (Asian, African American, White, and Hispanic) and three regional locations (New York City, Cincinnati, and the San Francisco Bay area of California). Urinary concentrations of TCS for all sites were between 1.6-956.0  $\mu\text{g l}^{-1}$ , with a median concentration of 7.2  $\mu\text{g l}^{-1}$ .

### 2.6.2 Plasma

TCS was detected in human plasma in a study investigating the body burden of phenolic halogenated compounds (PHC) (Hovander et al., 2002). Ten samples of blood plasma were randomly selected from male donors between the ages of 30 and 40, donated from a blood donor central at a hospital in Stockholm, Sweden. The authors identified TCS as one of more than 100 PHC's present in the plasma of Swedish males. Building on the work of Hovander et al. (2002), later studies, including the study of baseline plasma levels and urine excretion of TCS (Sandborgh-Eglund et al., 2006, see section 4.1), have detected the antimicrobial in humans.

The ubiquitous presence of TCS in the plasma of nursing mothers in Sweden has been documented by Allmyr et al. (2006). Plasma concentrations of TCS ranged from 0.010–38 ng g<sup>-1</sup>, and in contrast to the study by Sandborgh-Eglund et al. (2006), median TCS concentrations in subjects classified as users of products containing TCS were significantly higher than those in the control group, although the antimicrobial was detected in plasma samples from both the exposed and the control group. Of interest is the presence of TCS in the entire study population, indicating that other routes of exposure than personal care products influence plasma concentrations of TCS. Determining the various potential sources of TCS in human tissues warrants further research. As it currently stands, not all countries require TCS to be listed on a product's label, making it difficult to ascertain all the potential sources of exposure to the antimicrobial.

The influence of age, gender, and place of residence on plasma concentrations of TCS in the Australian population have been examined by Allmyr et al. (2007). In this particular study, place of residence had no effect on serum concentrations of TCS, while age and gender exerted minimal yet significant influence. TCS concentrations were more elevated in males than females, and reached peak concentrations in the group of 31-45 year old males and females. Due to the lack of marked differences in plasma concentrations observed in the study, the authors concluded that the exposure of the Australian population to TCS is relatively homogenous. In comparison to data from the Swedish population, serum levels of TCS were 2 times higher in the Australian

population, a phenomenon which is most likely due to the fact that the Swedish government strongly discourages consumers from using antibacterial products (Swedish Chemical Agency, 2001).

Dirtu et al. (2008) tested the sensitivity of solid-phase extraction and gas chromatography coupled to electron-capture negative-ionization mass spectrometry to detect phenolic compounds, including TCS, in human serum. The method used in the study yielded results that were comparable to previous data collected on the levels of TCS in human fluids. In this study, the median concentration of TCS in Belgian human serum samples (n=21) was 0.52 ng ml<sup>-1</sup>. As in earlier studies, TCS exhibited a high detection frequency as was evidenced by its presence in all the samples.

### *2.6.3 Breast milk*

The lipophilicity of TCS ( $K_{ow}=4.76$ ) coupled with its relative stability in human tissues makes it probable that the antimicrobial will be present in breast milk, with concentrations relating to maternal serum levels and the fat content of the milk (Ito and Lee, 2003). The presence of TCS in the breast milk of Swedish women was first reported by Adolfsson-Erici et al. (2002). A second study measuring TCS in breast milk was carried out by Allmyr et al. (2006), confirming the findings that were initially reported Adolfsson-Erici et al. (2002). Concentrations of TCS in milk samples were higher in women who used personal care products containing TCS, compared to women who did not, although TCS and/or its metabolites were detected in all the milk samples. Concentrations of TCS ranged from <0.018 to 0.95 ng g<sup>-1</sup>, which is comparable to the concentrations measured by Adolfsson-Erici et al. (2002). The universal presence of TCS in both groups indicates that personal care products are not the only source of human exposure to the antimicrobial. The levels of TCS in the subject's milk were significantly lower than levels measured in their plasma, indicating that infants receive a smaller dose of TCS than what is present in maternal systemic circulation.

Building on the landmark study conducted by Adolsson-Erici et al. (2002), a risk assessment for TCS in human breast milk was conducted by Dayan (2007). The study obtained 62 samples of breast milk from The Mothers Milk Banks in California and Texas. A GC/MS method was used to measure TCS levels in the milk samples, expressing the results as TCS per lipid basis. The results of the study were as follows: no TCS detected in 2 of the samples, trace amounts present in 9 of the samples, with the remaining 51 samples ranging in TCS concentrations from 100-2100  $\mu\text{g kg}^{-1}$  lipid. Based on the finding that a 6500-fold margin of safety exists between the levels of human exposure and the highest concentration of TCS that would elicit any adverse effects in human systems, the author concluded that the levels of TCS measured in breast milk do not pose a risk to breastfeeding infants. Interestingly, a recent review on TCS and development of margins of safety for consumer products by Rodrick et al. (2010) did not assess the exposure of infants to TCS through breast milk and the associated risks. The prevalence of TCS in human systems warrants further investigation into the bioaccumulation potential and toxicological effects of the antimicrobial, especially during sensitive periods of fetal and neonatal development.

## **2.7 Kinetics and Metabolism**

### *2.7.1 Dermal*

The percutaneous absorption of TCS from personal care product preparations was first investigated by Black et al. (1975). Rat skin was treated with shampoo or aerosol deodorant containing 0.05% (w/v) and 0.1% (w/v) [ $^3\text{H}$ ] TCS, respectively. The degree of TCS penetration was calculated from the amount of radioactivity excreted from the animals. Of the total amount of shampoo and aerosol deodorant applied, the bulk of TCS was removed by rinsing, with only small amounts penetrating the skin. Kanetoshi et al. (1992) confirmed that in mice, TCS is absorbed through the skin and is widely distributed throughout the various compartments in the body. Tissue concentrations of TCS peaked at 12 or 18 h and were present in decreasing order in the following tissues: gall bladder, liver, lung, adipose tissue, and blood. Moss et al. (2000) used a

similar approach to characterize the metabolism and kinetics of TCS. Following a dermal application of  $^3\text{H}$ -labelled TCS to the backs of female rats, the antimicrobial penetrated the dermis within the first hour of the experiment and was subsequently removed from the bloodstream. The primary elimination route of radioactivity was through fecal matter, with urinary excretion constituting a secondary removal pathway. In both urine and feces, TCS glucuronide and sulphate were detected, indicating that phase II biotransformation reactions play an essential role in the metabolism of the antimicrobial. Moreover, TCS glucuronide and sulphate were extracted from rat skin *in vivo*, suggesting that the antimicrobial is locally metabolised in skin cells. The amount of TCS that entered systemic circulation over the 24 hour period was 21%; 12% of radioactivity was in the feces, 8% in the carcass, 1% in the urine, 30% in the stratum corneum, with 26% remaining on the surface of the skin.

Queckenberg et al. (2010) characterized the absorption and pharmacokinetics of TCS after a dermal administration in human subjects. A hydrophobic cream containing 2% TCS was applied to the skin of six Caucasian volunteers. The 12 h exposure period culminated by the subjects taking a shower to eliminate any cream that remained on their skin. Urinary excretion of free and conjugated TCS was measured in intervals up to 168 h post-application. Of the TCS absorbed, the majority of the antimicrobial was excreted within 24 h. The half-life of TCS was calculated to be 10.8 h. This value is consistent with the previous study by Sandborgh-Englund et al. (2006), which determined the median half-life of TCS based on urinary excretion to be 11 h, following an oral administration. The total amount of TCS excreted is reflective of the amount absorbed, indicating a limited potential for accumulation in the body, and further reinforcing previous findings that in humans, urinary excretion is a major elimination route for the antimicrobial.

### *2.7.2 Subcutaneous*

To investigate the kinetics of subcutaneous exposure, female rats were injected with 0.5 ml of [ $^3\text{H}$ ] TCS solution in aqueous polyethylene glycol (Black et al., 1975). The animals were

housed in individual metabolic cages, and urine and feces were collected for the analysis of radioactivity. Within 4 days of the injection, 89.2% of the dose was recovered, with 33% of TCS recovered in urine. In agreement with other animal studies on the pharmacokinetics of TCS, a greater proportion of radioactivity was eliminated in feces than urine. TCS levels in the blood peaked at 6 hours after the administration of the dose, decreasing steadily after this time, and the biological half-life was calculated as 14 hours.

### 2.7.3 Oral

The earliest published kinetic study of orally administered TCS is the study by Tulp et al. (1979). A single oral dose of 500 mg kg<sup>-1</sup> TCS was given to male albino Wistar rats housed in metabolic cages for 7 days. Fecal matter and urine was collected daily and upon termination of the experiment, liver and abdominal fat were collected for analysis. TCS was metabolised primarily through hydroxylation, with scission of the ether bond representing a minor biotransformation pathway. Five hydroxylated metabolites were detected in urine, whereas only three of these metabolites were present in feces. The metabolite 2,4-dichlorophenol was detected in both urine and feces, with 4-chlorocatechol occurring in urine only, both of these metabolites being the product of the scission of the ether bond. In feces, TCS and its metabolites were excreted primarily unconjugated, with significant amounts of the parent compound present in both urine and feces. On completion of the experiment (7 days), TCS was present in both liver and abdominal fat samples, but because the dose of the TCS was so high (500mg kg<sup>-1</sup>), no conclusions could be made about its bioaccumulation potential. The authors concluded that the metabolism of TCS is unlikely to yield chlorodibenzo-*p*-dioxins or chlorodibenzofurans.

A later study by Kanetoshi et al. (1988) examined the disposition and excretion of TCS and its three chlorinated derivatives in mice. [<sup>3</sup>H]-TCS and 2',3,4,4'-tetrachloro-2-hydroxydiphenyl ether, 2',4,4',5-tetrachloro-2 hydroxydiphenyl ether and 2',3,4,4',5-pentachloro-2-hydroxydiphenyl ether were orally administered to male mice. Radioactivity was primarily distributed in the gall bladder, liver, lung, heart, and kidneys. Of the five tissues, TCS was most



concentrated in the gall bladder, which the authors attributed to biliary excretion via enterohepatic circulation. These results are consistent with other studies that have determined that in animals, radiolabelled TCS is excreted primarily in feces and secondarily in urine (Siddiqui and Buttar, 1979; Moss et al., 2000). The  $^3\text{H}$ -labelled TCS was rapidly absorbed and excreted, with calculated half-life of 8 hours. At the 24 hour mark, the radioactivities of TCS and its three chlorinated derivatives were nearly completely eliminated from all tissues, indicating that TCS and/or its metabolites do not accumulate in the body. Gilbert and Williams (1987) investigated the oral retention and pharmacokinetics of [ $^3\text{H}$ ]-TCS in an antimicrobial toothpaste. Twelve healthy male volunteers between the ages of 19-37 were recruited for the study and brushed their teeth with 1g of toothpaste containing 0.02% of [ $^3\text{H}$ ]-TCS. The oral retention of TCS was found to be  $36.3 \pm 1.4\%$ . TCS remained in bacterial plaque for at least 8 hours after dosage and in oral mucosa for three hours. A review on the safety of TCS was conducted by DeSalva et al. (1989), evaluating data from multiple sources including pre-clinical and clinical studies, data submitted to the Antimicrobial I OTC Review Panel and unpublished work from the Pharmacology and Toxicology Department of the Colgate-Palmolive company. Humans, dogs, rabbits, and rats were used to study the pharmacokinetics of TCS. Routes of administration included oral, dermal, and intravenous; pharmacokinetic data on the antimicrobial indicated that in humans, the kidneys are the main excretory organ responsible for the elimination of TCS, as is evidenced by the proportion of TCS and its conjugates that are concentrated in urine.

The buccal absorption of TCS from 0.03% mouthwash was calculated by Lin (2000). Subjects were given 15 ml of TCS oral mouthrinse or a placebo oral rinse to be used twice daily. Blood and dental plaque samples were collected 4 hours and 1 hour after rinsing, respectively. The average daily retention dose of TCS was 0.660 mg which translates into 7.33% of the original dose. On average, TCS concentrations in plaque were in the range of 20.5-46.4  $\mu\text{g}$  per g of plaque collected. The average concentration of TCS in plasma ranged from 74.5-94.2  $\mu\text{g ml}^{-1}$ , with peak concentrations attained two days following exposure. Plasma levels of TCS returned to

baseline concentrations eight days after the last treatment. More recently, Sanborgh-Englund et al. (2006) examined the pharmacokinetic pattern of TCS in humans after a single dose oral administration. Subjects were required to fast overnight and the following morning they were given 13 ml of a 0.03% mouthwash solution, equivalent to a 4 mg oral dose of TCS. Blood and urine levels were monitored prior to exposure and up to 8 days after exposure, and baseline levels of TCS in plasma and urine were determined for each subject. Plasma concentrations of TCS increased rapidly after dosing, attaining peak levels within 1 to 3 hours, resulting in a terminal plasma half-life of 21 hours. In plasma, 30-35% of TCS was present in the unconjugated form. These results are different from those of DeSalva et al. (1989), who reported that the entirety of TCS measured in plasma was in the conjugated form (either glucuronide or sulfonate). Unfortunately, the basis for these differences is unknown. Lastly, the cumulative urinary excretion of TCS was 54%, occurring 4 days after exposure and the calculated urinary excretion half-life was 11 hours. The results of this study confirmed again that TCS is rapidly absorbed from the gastrointestinal tract and swiftly eliminated from the body, usually within a 24 hour period.

#### *2.7.4 Intravenous*

Siddiqui and Buttar (1979) investigated the pharmacokinetics of intravenous and intravaginal injections of TCS in sexually mature virgin Wistar rats.  $^{14}\text{C}$ -Triclosan was injected into the femoral vein (5 mg kg<sup>-1</sup> in polyethylene glycol-400) or the vaginal orifice (5 mg kg<sup>-1</sup> in corn oil) and liquid scintillation spectrophotometry was used to determine radioactivity. In both treatments, the rate of transfer from plasma to tissues was rapid, likely attributable to the lipophilicity of TCS. The half-life of TCS in the  $\beta$  phase was 8.8  $\pm$  0.6 hr and the blood clearance rate was 77.5  $\pm$  11.3 ml kg<sup>-1</sup> hr<sup>-1</sup> after intravenous injection or intravaginal administration. In the intravenous administration, after 24 hours, 18% of TCS was excreted in feces and 9% was eliminated in urine. In contrast, the intravaginal injections resulted in excretions of 26% in feces and 14% in urine.

### 2.7.5 Phase I and II enzymes

There is evidence for the ability of TCS to interact with cytochrome P450 enzymes in liver microsomes, although the effects may be dose- and species-dependent. The antimicrobial inhibited in vitro methylcholanthrene (MC)- and phenobarbital (PB)-inducible P450-dependent monooxygenases, specifically pentoxyresorufin O-depentylase (PROD) and ethoxyresorufin O-deethylase (EROD) activity, competitively or noncompetitively (Hanioka et al., 1996). These results are important since induction of P450 isoforms of the CYP1A or CYP2B subfamily closely relates to toxicity of the antimicrobial. A later study (Hanioka et al., 1997) reported that TCS (Irgasan DP300) induces the P450 isoforms of the CYP2B subfamily. Similar results suggesting that TCS is a phenobarbital-type inducer were reported by Jinno et al. (1997), in cultured rat hepatocytes and by Katenoshi et al. (1992), in mice liver microsomes. In contrast, Ishibashi et al. (2004) found no evidence that TCS induces EROD and PROD activity in the hepatic microsomes of female medaka, *Oryzias latipes*. This discrepancy may be due to physiological differences between mammals and fish, and/or differences in exposure. Jacobs et al. (2005) presented in vitro evidence for TCS acting as a ligand, with a moderate affinity for the human pregnane X receptor (hPXR). The hPXR regulates the expression of phase I enzymes such as cytochrome P450 3A4 (CYP3A4), which play an integral role in the biotransformation of approximately 50% of pharmaceuticals (Luo et al., 2002; Jacobs et al., 2005). Compounds that are capable of upregulating the transcription of CYP3A4 enzymes can alter the rate at which pharmaceuticals are metabolised, creating the potential for adverse effects.

TCS, structurally similar to hydroxylated polychlorinated biphenyls, also interacts with Phase II enzymes. TCS is both a substrate and inhibitor of sulfonation and glucuronidation in human liver cytosol and microsomes (Wang et al., 2004). The inhibition of sulfonation was non-competitive, while the inhibition of glucuronidation was competitive. These results confirm earlier evidence of the inhibition of sulfation, specifically that of thyroid hormones, by TCS and other hydroxylated halogenated chemicals (Schoor et al., 1998). TCS sulfonation in polar bear

liver, similar to human liver in respect to enzyme affinity, was characterized by Sacco and James (2005). In addition to effects on Phase I and Phase II enzymes, TCS may have direct effects on mitochondria, impairing function through an uncoupler effect and disrupting mitochondrial membrane fluidity (Newton et al., 2005).

The available evidence indicates that the two most probable routes of exposure to TCS in humans are ingestion and/or percutaneous absorption. Low but detectable levels of TCS have been reported in drinking water (Loraine and Pettigrove, 2006; Servos et al., 2007). Blanset et al. (2007) estimated the ADI (acceptable daily intake) for TCS at  $0.05 \text{ mg kg}^{-1} \cdot \text{day}^{-1}$ , and concluded that based on TCS levels typically measured in drinking water, the risk to human health is minimal. Concentrations of TCS in the blood are directly related to consumer use patterns of the antimicrobial. In humans, after an oral dose of TCS, the antimicrobial is eliminated primarily as conjugated metabolites in urine. In the study by Sandborough et al. (2006), approximately 70% of the total amount of TCS measured in plasma existed as either sulphate or glucuronide conjugates. TCS interacts with phase I and II enzymes, contributing to its toxicity and endocrine disrupting properties.

## **2.8 Toxicity**

Numerous studies have evaluated the toxicity of TCS in various organisms, including algae, invertebrates, amphibians, fish, birds and mammals. Data from mammalian studies, including humans, has recently been reviewed by Rodricks et al. (2010). The following section briefly summarizes those studies and provides a more detailed review of toxicity data from non-mammalian species.

### *2.8.1 Acute Toxicity*

#### 2.8.1.1 Terrestrial organisms

Several recent studies investigated the terrestrial ecotoxicological effects of TCS. TCS inhibited plant growth ( $EC_{50}$  57-108  $\text{mg kg}^{-1}$ ) and soil respiration, with some evidence for recovery after 2 days, possibly linked to degradation of TCS (Liu et al., 2009). Waller and

Kookana (2009) reported that TCS, at concentrations below 10 mg kg<sup>-1</sup>, disturbs the nitrogen cycle in some soils. An ecological risk assessment for TCS in the terrestrial environment has been published by Reiss et al. (2009). The assessment reviewed available data and found satisfactory margins of safety for terrestrial organisms, including earthworms, plants, and soil microorganisms exposed to TCS in soils amended with sewage sludge, and to birds and mammals exposed indirectly through the consumption of earthworms and fish. However, the number of studies available for the risk assessment was relatively small (n=31), indicating that further investigations of the potential impact of TCS on the terrestrial ecosystems are needed.

#### 2.8.1.2 Aquatic organisms

Microorganisms and algae- TCS is primarily a water-borne pollutant, with numerous studies having investigated its toxicity in aquatic organisms (Table 2.3). The toxicity of TCS to WWTP sludge organisms, algae, daphnids, and fish was assessed by Orvos et al. (2002). While sludge microorganisms were unaffected by TCS, the aquatic species that appeared most vulnerable to the toxic effects of TCS were algal species such as *Scenedesmus subspicatus*, with a 96-h biomass EC<sub>50</sub> (median effective concentration) of 1.4 µg l<sup>-1</sup> and a 96-h No-Observed-Effect-Concentration (NOEC) of 0.69 µg l<sup>-1</sup> (Orvos et al., 2002). Similar evidence regarding algal sensitivity was provided by Tatarazako et al. (2004) for *Selenastrum capricornutum*, and by DeLorenzo and Fleming (2008) for a marine phytoplankton, *Dunaliella tertiolecta*, (Table 2.3). The toxic effects of TCS were primarily due to the neutral form of TCS, where sorption and ionization could potentially temper these effects in the aquatic ecosystems. The lowest NOEC for algae, integral components of aquatic food webs, is less than 1 µg l<sup>-1</sup>, with TCS being measured in the range of 0.2-2.7 µg l<sup>-1</sup> in U.S. wastewater effluent (Reiss et al., 2002). It is then possible that current levels of TCS in rivers and streams (PEC, Predicted Environmental Concentration) may surpass the NOEC for algae, as indicated by HQ value (Hazard Quotient, PEC divided by NOEC) greater than 1. Coogan et al. (2007) measured TCS levels in the algal species, *Cladophora* spp., located downstream from a WWTP. Although TCS concentrations in the water

column decreased downstream from the effluent output ( $0.12 - 0.06 \mu\text{g l}^{-1}$ ), concentrations of the antimicrobial in algae demonstrated the reverse pattern ( $100 - 150 \mu\text{g l}^{-1}$ ), indicating bioaccumulation. It is unclear how the propensity of algae to accumulate TCS affects the species vulnerability to the toxicity of the antimicrobial.

Invertebrates- Aquatic invertebrates also exhibit vulnerability to TCS (Table 2.3). Short term (30 min) exposure of hemocytes, the immune cells of bivalve *Mytilus galloprovincialis*, to TCS reduced lysosomal stability and induced the release of lysosomal hydrolytic enzymes (Canesi et al., 2007). Moreover, in vivo exposures of the bivalve affected glycolytic enzymes and redox balance in different systems/organs. For *Daphnia magna*, a key invertebrate aquatic species, the 48-h median effective concentration was  $390 \mu\text{g l}^{-1}$  (Orvos et al., 2002). The toxicity of TCS to the midge, *Chironomus tentans*, and the freshwater amphipod, *Hyalella azteca*, was evaluated in a 10-day exposure test ( $\text{LC}_{50}$   $0.4 \text{ mg l}^{-1}$  and  $0.2 \text{ mg l}^{-1}$ , respectively). TCS was more toxic than carbamazepine, an anticonvulsant, and atorvastatin, a lipid regulator (Dussault et al., 2008).

Fish- Kim et al. (2009) assessed the acute toxicity of TCS in two test species, a freshwater crustacean (*Thamnocephalus platyurus*) and a fish (*Oryzias latipes*). The organisms were exposed to a range of TCS concentrations and the 24-h  $\text{LC}_{50}$  values were determined by probit analysis. The  $\text{LC}_{50}$  values of TCS for *T. Platyurus* and *O. Latipes* were  $0.47 \text{ mg l}^{-1}$  and  $0.60 \text{ mg l}^{-1}$ , respectively. The  $\text{LC}_{50}$  (96-h) value for *O. Latipes* calculated in this study confirms the previous findings of Tarazako et al. (2004), in which an  $\text{LC}_{50}$  value of  $0.40 \text{ mg l}^{-1}$  was ascertained for the antimicrobial.

Nassef et al. (2010) applied in *ovo* nanoinjection of TCS to medaka embryos and determined  $4.2 \text{ ng egg}^{-1}$  as the  $\text{EC}_{50}$  value based on survival and embryonic development. Foran et al. (2000) examined the acute toxicity of TCS in medaka fry (Table 2.3). Concentrations of  $1 \text{ mg l}^{-1}$  and  $500 \mu\text{g l}^{-1}$  resulted in fry death within 24 hours and 3 days, respectively. The  $\text{LC}_{50}$  (48-h) for medaka fry was calculated to be  $352 \mu\text{g l}^{-1}$ . A 96-h median lethal concentration of  $602 \mu\text{g l}^{-1}$

$\text{l}^{-1}$  for medaka fry was reported by Ishibashi et al. (2004). Although this value is higher than the  $\text{LC}_{50}$  (48-h) of  $352 \mu\text{g l}^{-1}$  previously determined by Foran et al. (2000), it appears that during early development, fish are especially vulnerable to the toxic effects of TCS. Nassef et al. (2009) used adult Japanese medaka as the test organism in an acute toxicity study and observed concentration- and time-dependent mortality. Test solutions of TCS were 1, 2, 2.4, and 3  $\text{mg l}^{-1}$  and the adult fish were exposed to TCS for a period of 96 h. Reported 96-h survival rates were 100% ( $1\text{mg l}^{-1}$ ), 16.7% ( $2 \text{ mg l}^{-1}$ ), 3.3% ( $2.4 \text{ mg l}^{-1}$ ), and 0% ( $3 \text{ mg l}^{-1}$ ). At higher exposure levels ( $2.4$  and  $3 \text{ mg l}^{-1}$ ), fish displayed abnormal behaviours and experienced a loss of equilibrium. The  $\text{LC}_{50}$  (96-h) of TCS for adult medaka was  $1.7 \text{ mg l}^{-1}$ , while the NOEC was estimated at  $1.7 \mu\text{g l}^{-1}$ , which is 12 times higher than the PEC for the antimicrobial. The authors of this study are in agreement with Ishibashi et al. (2004), in their conclusion that TCS is highly toxic to fish.

Oliveira et al. (2009), in an experiment similar to the one by Nassef et al. (2009), studied the acute toxicity of TCS in different life stages of zebrafish (*Danio rerio*). The effect of TCS on mortality, and developmental, genetic and enzymatic biomarkers were determined in adult fish and embryo/larvae using the OECD guidelines on Fish Embryo Toxicity. At concentrations above  $0.7 \text{ mg l}^{-1}$ , TCS exhibited teratogenic effects, delaying embryo development and resulting in mortality within 48 hours. The  $\text{LC}_{50}$  (96-h) of TCS for embryo/larvae was  $0.42 \text{ mg l}^{-1}$ . The results of the biomarker analysis indicated that TCS increased the activity of ChE ( $0.25 \text{ mg l}^{-1}$ ), LDH ( $0.25 \text{ mg l}^{-1}$ ), and GST ( $0.25$  and  $0.35 \text{ mg l}^{-1}$ ). Based on the results, concentrations of TCS equal to, or above  $0.3 \text{ mg l}^{-1}$ , were estimated to constitute a hazard for aquatic ecosystems. Using the OECD Guideline TG 203 in semi-static conditions, the  $\text{LC}_{50}$  (96-h) value for adult zebrafish was determined as  $0.34 \text{ mg l}^{-1}$ , a value similar to the  $\text{LC}_{50}$  (96-h) of  $0.42 \text{ mg l}^{-1}$  for zebrafish larvae. The acute toxicity of TCS was primarily limited to behavioural effects, none of which were studied in detail. Abnormal behavioural patterns observed during the study included irregular swimming, loss of equilibrium, and anomalous gill movement. In contrast to the embryo/larvae stage, there was no evidence of genotoxicity or changes in enzyme levels of ChE, GST, and LDH

in adult zebrafish. Although there are differences in species sensitivity to TCS, the range of LC<sub>50</sub> (96-h) is relatively small (Table 2.3); the 96-h median lethal concentration values for *Pimephales promelas* and *Lepomis macrochirus* were 260 and 370 µg l<sup>-1</sup>, respectively (Orvos et al., 2002), while the value for adult zebrafish was 340 µg l<sup>-1</sup>. During early development, juvenile fish are more sensitive to TCS than adults. In an early life-stage toxicity test with *Oncorhynchus mykiss*, the NOEC and the lowest-observed-effect concentration (LOEC) were 34.1 µg l<sup>-1</sup> and 71.3 µg l<sup>-1</sup>, respectively (Orvos et al., 2002).



**Table 2.3:** Effects of Triclosan (TCS) in freshwater (FW) and marine (SW) organisms

Test species	Life stage	System type	Route of exposure	Test duration	TCS exposure	Endpoint	Reference
Phytoplankton ( <i>Dunaliella tertiolecta</i> )		SW	Water (static)	Acute (96-h)	3.5 µg l <sup>-1</sup>	EC <sub>50</sub> (population density)	deLorenzo and Fleming, 2008
Green alga ( <i>Selenastrum capricornutum</i> )		FW	Water (static)	Acute (72-h)	4.7 µg l <sup>-1</sup>	EC <sub>50</sub> (growth)	Tarazako et al. 2004
Green alga ( <i>Scenedesmus subspicatus</i> )		FW	Water (static)	Acute (96-h)	1.4 µg l <sup>-1</sup>	EC <sub>50</sub> (biomass)	Orvos et al. 2002
Alga ( <i>Closterium ehrenbergii</i> )		FW	Water (static)	Acute (48-h)	620 µg l <sup>-1</sup> ; 250 µg l <sup>-1</sup>	EC <sub>50</sub> Genotoxicity	Ciniglia et al. 2005
Blue-green alga ( <i>Anabaena flos-aquae</i> )		FW	Water (static)	Acute (96-h)	1.6 µg l <sup>-1</sup>	EC <sub>50</sub> (biomass)	Orvos et al. 2002
<b>INVERTEBRATES</b>							
<i>Daphnia magna</i>		FW	Water (renewal)	Acute (48-h) 21 d	390 µg l <sup>-1</sup> 40 µg l <sup>-1</sup>	EC <sub>50</sub> NOEC reproduction	Orvos et al. 2002
<i>Ceriodaphnia dubia</i>		FW	Water (renewal)	Acute (48-h) 7 d	240 µg l <sup>-1</sup> 182 µg l <sup>-1</sup>	EC <sub>50</sub> NOEC reproduction	Orvos et al. 2002
		FW	Water (renewal)	6-7 d	220 µg l <sup>-1</sup>	IC <sub>50</sub> (growth)	Tarazako et al. 2004
<i>Chironomus tentans</i> <i>Hyalella azteca</i>		FW	Water (renewal)	10 d	400 µg l <sup>-1</sup> 200 µg l <sup>-1</sup>	LC <sub>50</sub>	Dussault et al. 2008
Grass shrimp	Embryo	SW	Water	Acute	651 µg l <sup>-1</sup>	LC <sub>50</sub>	DeLorenzo et al.

<i>(Palaemonetes pugio)</i>	Larvae Adult		(renewal)	(96-h)	154 $\mu\text{g l}^{-1}$ 305 $\mu\text{g l}^{-1}$	LC <sub>50</sub> LC <sub>50</sub>	2008
Crustacean <i>(Thamnocephalus platyurus)</i>		FW	Water (static)	Acute (24-h)	470 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	Kim et al. 2009
Bivalve <i>(Mytilus galloprovincialis)</i>	hemocytes	SW	In vitro	Acute (30 min)	1 $\mu\text{M}$	↓ lysosomal membrane stability	Canesi et al. 2007
	Whole animal	SW	Injection	Acute (24-h)	2.9 $\text{ng g}^{-1}$	Altered hemocyte and digestive gland function	Canesi et al. 2007
Zebra mussel <i>(Dreissena polymorpha)</i>	hemocytes	FW	In vitro	Acute (60 min)	0.1 $\mu\text{M}$	Genotoxicity	Binelli et al. 2009a, b
			In vivo	Acute (96-h)	1 nM	Genotoxicity	
Rainbow trout <i>(Oncorhynchus mykiss)</i>	Adult	FW	Water (flow- through)	Acute (96-h)	390 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	CIBA, 1998
	embryo	FW		61 d 35 d	71.3 $\mu\text{g l}^{-1}$	Delayed swim- up ; ↓ 35-dph survival; erratic swimming, locked jaw	Orvos et al. 2002
Medaka <i>(Oryzias latipes)</i>	Fertilized eggs	FW	Water (renewal)	14 d	313 $\mu\text{g l}^{-1}$	↓ hatching; delayed hatching	Ishibashi et al. 2004
	Larvae (24-h old)			Acute (96-h)	602 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	

	Male fish			21 d	20 $\mu\text{g l}^{-1}$	$\uparrow$ liver Vtg	
	fry	FW		Acute (48-h)	350 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	Foran et al. 2000
	eggs	FW SW	in ovo injection	14 d 1 d post- fertilization	400 $\mu\text{g l}^{-1}$ 4.2 ng egg <sup>-1</sup>	IC <sub>50</sub> (hatching) EC <sub>50</sub> (survival)	Tarazako et al. 2004 Nasseff et al. 2010
	larvae	FW	(static)	Acute (96-h)	600 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	Kim et al. 2009
	adult	SW	(renewal)	Acute (96-h)	1700 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	Nassef et al. 2009
Bluegill sunfish ( <i>Lepomis macrochirus</i> )		FW	Water (renewal)	Acute (96-h)	370 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	Orvos et al. 2002
Fathead minnow ( <i>Pimephales promelas</i> )	Adult	FW	Water (renewal)	Acute (96-h)	260 $\mu\text{g l}^{-1}$	LC <sub>50</sub> (at pH 7.5)	Orvos et al. 2002
	Full cycle	FW	Water (TCS in mixture)		0.1 and 0.3 $\mu\text{g l}^{-1}$ mixture of products	No effects F <sub>0</sub> ; $\uparrow$ larval deformities in F <sub>1</sub>	Parrott and Bennie, 2005
Zebrafish ( <i>Danio rerio</i> )	eggs	FW	Water (renewal)	9 d	220 $\mu\text{g l}^{-1}$	IC <sub>50</sub> (hatching)	Tarazako et al. 2004
	embryo	FW	24-well Micro plates	Acute (96-h)	420 $\mu\text{g l}^{-1}$	LC <sub>50</sub> ; teratogenic effects	Oliveira et al. 2009
	Adult	FW	Semi- static	Acute (96-h)	340 $\mu\text{g l}^{-1}$	LC <sub>50</sub>	Oliveira et al. 2009
<b>AMPHIBIANS</b>							
Bullfrog	tadpoles	FW	water	Acute (96 h)	0.15 $\mu\text{g l}^{-1}$	$\uparrow$ hindlimb	Veldhoen et al.

<i>(Rana catesbeiana)</i>						development, ↓ body weight, disruption of thyroid hormone- associated gene expression	2006				
<i>Xenopus laevis</i>	XTC-2 cells	FW	In vitro	Acute (24 h)	0.03 µg l <sup>-1</sup>	Altered thyroid hormone receptor mRNA expression	Veldhoen et al. 2006				
<i>Acris crepitans blanchardii</i>	Larvae	FW	water	Acute (96-h)	367 µg l <sup>-1</sup>	LC <sub>50</sub>	Palenske and Dzialowski, 2010				
<i>Bufo woodhousii woodhousii</i>	Stage 30				152 µg l <sup>-1</sup>						
<i>Rana sphenoccephala</i>	Stage 41				562 µg l <sup>-1</sup>						
<i>Xenopus laevis</i>	Stage 41				343 µg l <sup>-1</sup>						
Leopard frog ( <i>Rana pipiens</i> )	tadpoles	FW	Static (weekly renewal)	24 d	0.23 µg l <sup>-1</sup>	↓activity, loss of startle response, ↓ survivorship	Fraker and Smith, 2004				
American toad ( <i>Bufo americanus</i> )	tadpoles	FW	Static (weekly renewal)	14 d	230 µg l <sup>-1</sup>	↑ activity	Smith and Burgett, 2005				
EC50	–	Effective	concentration;	LC50-Lethal	concentration;	NOEC	–	No	Observed	Effect	Concentration

### 2.8.1.3 Amphibians

The effects of TCS were also investigated in amphibians. There is evidence to indicate that TCS affects behaviour and survivorship in tadpoles, however the effects seem to be species-specific. TCS increased activity levels in American toad tadpoles, *Bufo americanus*, although the effects on survivorship were not concentration dependent (Smith and Burgett, 2005). In tadpoles of the Northern leopard frog, *Rana pipiens*, ecologically relevant concentrations of TCS decreased activity and survivorship (Fraker and Smith, 2004), indicating that the HQ for this specific endpoint is  $>1.0$ . In the latter study, no evidence of an interaction between TCS and caffeine or acetaminophen, pharmaceuticals often co-occurring in WWTP effluents, was detected. Palenske and Dzialowski (2010) assessed the species specific and developmental toxicity of TCS in amphibian larvae for *Acris crepitans blanchardii*, *Bufo woodhousii woodhousii*, *Rana sphenoccephala*, and *Xenopus laevis*. Bioconcentration factors for *X. laevis*, *B. woodhousii woodhousii*, and *R. sphenoccephala* were also determined. As is the case with other aquatic species, TCS toxicity was dependent upon larval maturity and amphibian species. *X. laevis* larvae were most vulnerable to TCS during the first two developmental stages. Larval LC<sub>50</sub> values were reported as follows: 259-664  $\mu\text{g l}^{-1}$  (*X. laevis*), 367  $\mu\text{g l}^{-1}$  (*A. crepitans blanchardii*), 152  $\mu\text{g l}^{-1}$  (*B. woodhousii woodhousii*), and 562  $\mu\text{g l}^{-1}$  (*R. sphenoccephala*), with significant differences observed for all three amphibian species (Table 2.3). In this study, TCS tissue uptake was related to larval species, stage of development and mean mass. Bioconcentration factors ranged from 44 in *X. laevis* up to 740 in *B. woodhousii woodhousii*.

### 2.8.1.4 Mammals

The toxicity of TCS has been tested in laboratory rodents, and other mammalian models. An early study by Lyman and Furia (1969), sanctioned by the Geigy Chemical Corporation, provided toxicological data on TCS in rats, concluding that TCS was neither acutely toxic (LD50 oral  $> 1\ 000\ \text{mg kg}^{-1}$ ) nor carcinogenic. Later, nephrotoxic effects of orally administered TCS in rats were reported by Chow et al. (1977), in a study where the accumulation of *p*-aminohippurate

(PAH) was estimated both in vivo and in vitro, using kidney slices to detect dose-related inhibition. A 1989 review on the safety of TCS was published by DeSalva et al. (1989). The majority of data in this review cited unpublished results from reports submitted by Ciba-Geigy Company to the Antimicrobial I OTC Review Panel. Ciba-Geigy Company tested the acute toxicity of TCS on four different species of animals; mouse, rat, rabbit, and dog. Based on these studies, TCS was deemed not to be an acute oral toxicant.

One of the more recent studies detailing the acute toxicity of TCS in mammals was conducted by Kanetoshi et al. (1992). The study evaluated the acute toxicity and percutaneous absorption of TCS and its chlorinated derivatives in male mice. The results indicated that TCS has a low acute toxicity ( $LD_{50} > 1\text{g kg}^{-1}$ ), a value which is in agreement with the previous findings of Lyman and Furia (1969). The authors previously reported that Irgasan DP300 is commonly detected in commercial textile products and that an exposure to sodium hypochlorite (domestic bleach), leads to the formation of three different chlorinated derivatives. Worth noting, is that the chlorinated derivatives of TCS are significantly more toxic than TCS itself, and as the number of chlorine substitutions in the TCS derivatives increases, their  $LD_{50}$  values decrease (Kanetoshi et al., 1992). Dermal contact with textiles that have TCS incorporated into their fibres may expose consumers to chlorinated TCS derivatives. However, Rodricks et al. (2010) provided a recent critical review of the current mammalian literature and developed margins of safety for consumer products, concluding that the exposure to TCS in consumer products is not expected to cause adverse health effects in humans.

Current literature suggests that while TCS is not acutely toxic to mammals, aquatic species such as algae and certain types of fish are highly sensitive to the antimicrobial. The NOEC for fish is in the range of 34.1-200  $\mu\text{g l}^{-1}$  (Orvos et al., 2002; Ishibashi et al., 2004; Capdevielle et al., 2008), a concentration range which exceeds the PECs (0.01 - 0.14  $\mu\text{g l}^{-1}$ , Table 2.2) for TCS. The toxicity of TCS is also dependent on the life stage of the organism, with juveniles having a tendency to be more vulnerable to the toxic effects of the antimicrobial.

### 2.8.2 Subacute/subchronic and chronic toxicity

Toxicity data from prolonged exposure studies of aquatic organisms to TCS are relatively scarce (Table 2.3). Algae were determined to be the most sensitive aquatic organisms in experiments lasting up to 21 days, as has been shown in the acute toxicity studies (Orvos et al., 2002). Significant adverse effects on survival and reproduction were also detected in the invertebrate, *Daphnia magna*. While juvenile rainbow trout were adversely affected by chronic exposures to TCS, fathead minnows were not. Parrott and Bennie (2009) used fathead minnow life-cycle tests to study the sub-lethal effects of environmentally relevant mixtures (ng l<sup>-1</sup> range) of a personal care product (TCS, up to 115 ng l<sup>-1</sup>) and six common pharmaceuticals. In comparison to controls, no significant differences in survival, growth, development, or reproduction were observed in any of the treatment groups. The only significant effect of the PPCP mixture was observed in the F1 generation; the 100 and 300 ng l<sup>-1</sup> PPCP mixture but not 1000 ng l<sup>-1</sup>, produced significant increases in the rates of deformities, including cardiac edema, spinal deformities and yolk-sac edema. The magnitude of the effect was however low since only doubling or tripling of the deformity rate compared to controls, was observed. The results of the study are noteworthy, as they indicate that the chronic exposure of fathead minnows to an environmentally relevant mixture of 7 PPCPs did not affect any of the parameters tested, with the exception of F1 larval deformities, where the impact was relatively minor.

Subacute and chronic toxicity data from mammalian species, including mice, rats, hamsters and baboons are extensive, and have been reviewed by Rodricks et al. (2010). For systemic toxicity, excluding endocrine disruption, the effects of TCS were primarily limited to changes in the liver and kidneys. TCS induced changes in liver weight, liver enzymes, liver hypertrophy, and increased peroxisome size and numbers. In rodent species, renal toxicity was evidenced by inflammation and tubular regeneration.

### 2.8.3 Genotoxicity

Genotoxicity and mutagenicity studies using classical prokaryotic and eukaryotic systems were reviewed by Rodricks et al. (2010). The available evidence led the authors to conclude that TCS is neither genotoxic nor mutagenic. However, there is some evidence to suggest that TCS may be genotoxic in certain types of organisms and/or cell types.

Acute toxicity experiments (96-hour exposure) using hemocytes from zebra mussels (*Dreissena polymorpha*) that were exposed to environmentally relevant concentrations of TCS (1, 2, 3 nM) provided evidence of genotoxicity after only 24 hours of exposure (Binelli et al., 2009a). The genotoxicity of TCS in hemocytes was evaluated with the single cell gel electrophoresis (SCGE) assay (also known as the Comet assay), the micronucleus assay, and the Halo test, a measure of the apoptotic frequencies, while cytotoxicity was assessed with the neutral red retention assay. The genetic damage accrued in the hemocytes was significant at all three concentrations of TCS, following a concentration-dependent and time-dependent pattern. The authors of this study concluded that the genotoxicity of TCS in zebra mussels was likely due to a combination of oxidative stress and/or a direct effect on DNA. In a follow up study (Binelli et al., 2009b), hemolymph from zebra mussels was extracted and then used to investigate TCS's potential for both cytotoxicity and genotoxicity. In this experiment, antimicrobial concentrations of 0.1, 0.15, 0.2, and 0.3  $\mu\text{M}$  caused extensive DNA damage in hemocytes, as was indicated by the SCGE assay and the apoptotic frequency. Although the range of concentrations used in this study was very narrow, the results clearly indicated that TCS's genotoxicity increased in a dose dependent fashion. Based on these two studies, there is compelling evidence to suggest that TCS has genotoxic effects in zebra mussels, both in vivo and in vitro, although future studies are needed to confirm these findings.

The genotoxicity of TCS has also been evaluated using the Comet Assay in the algal species *Closterium ehrenbergii* (Ciniglia et al., 2005). Algal cells were exposed to TCS for 96-h, at concentrations in the range of 0.125-1  $\text{mg l}^{-1}$ . At concentrations of 0.25  $\text{mg l}^{-1}$  and greater, the



genetic toxicity of TCS was apparent, with the antimicrobial exerting its toxicity in a dose dependent manner. Complete dissolution of the nucleus was observed at concentrations of 0.5 and 1 mg l<sup>-1</sup>. Although the results of this study indicate that TCS has genotoxic effects on *C. ehrenbergii*, the concentrations used in the experiment were much higher than those typically observed in surface water (HQ < 1.0), and as such, the results should be interpreted with caution.

The genotoxicity and cytotoxicity of TCS has been tested in animal cell lines. Zuckerbraun et al. (1998) demonstrated that TCS is cytotoxic to Smulow-Glickman cells (S-G cells), which are derived from a human gingival epithelial cell line. TCS damaged the integrity of the plasma membrane and induced apoptotic cell death. The effects of TCS on gingival cells are important, as TCS is a common ingredient in a number of oral hygiene products. Jirasripongpun et al. (2008) used the Comet Assay and the Apoptosis Assay to test the genotoxicity of TCS on two animal cell lines. KB and Vero cell lines were treated with two concentrations of TCS, the 50% inhibition concentration (IC<sub>50</sub>, 0.034 and 0.036 mM respectively) and the maximum concentration of TCS in personal care products (0.023 mM). In both cell lines, the number of comet cells increased as the concentration and exposure time to TCS increased. Most notably, genetic damage accrued from the exposure to TCS was observed at concentrations in the IC<sub>20-30</sub> range, following a 5 day exposure period. At levels of TCS that are normally in personal care products, the antimicrobial failed to produce any signs of genotoxicity. TCS is a lipophilic chemical and as such, could potentially accumulate in the body. However, exposure to TCS levels that cause genetic damage is unlikely, considering that pharmacokinetic studies have demonstrated that the antimicrobial is rapidly metabolized and readily eliminated from the body.

#### *2.8.4 Mutagenicity and carcinogenicity*

Reviews of data on TCS's safety by DaSalva et al. (1989), Bhargava and Leonard (1996), and more recently by Rodricks et al. (2010), included extensive data from mutagenicity assays, many completed by Ciba-Geigy Co. The overwhelming majority of these assays indicated that TCS did not exhibit any mutagenic potential, with the exception of the mammalian spot test. The

mammalian spot test was first performed by Fahrig et al. (1978) and resulted in a positive response, but later yielded negative results when repeated by Russell and Montgomery (1980). The study published by Fahrig was criticized, and it has been suggested that the effective dose of TCS used by Fahrig would result in maternal toxicity, thus precluding evaluation in the offspring. It is not clear why these two studies, using the same method, yielded such very different results. Fahrig et al. used a higher dose of TCS dissolved in HBSS, whereby Russell and Montgomery deemed TCS to be insoluble in HBSS, opting to dissolve the antimicrobial in methanol instead. Montgomery and Russell conjectured that due to the limited solubility of TCS in HBSS, the experiment by Fahrig et al. likely failed to inject any of the dams with TCS, which would explain the limited toxicity observed at the 50 mg kg<sup>-1</sup> dose, a dose which proved to be highly toxic to embryos in the study by Russell and Montgomery. The majority of researchers seem to accept the findings of the experiment by Russell and Montgomery, as reviews on TCS safety (DaSalva et al., 1989; Bhargava and Leonard, 1996; Rodricks et al., 2010) consistently conclude that TCS is not a mutagen and that personal care products containing the antimicrobial do not pose a risk to human health.

The mutagenic potential of TCS and its photodegradation products were later examined by Onodera et al. (1995), in two *Salmonella* strains tested with and without S9 fractions. Any mutagenic effects of TCS went undetected, due to the high toxicity of the antimicrobial to the test species. Following treatment with photo-irradiation and chlorine, TCS in aqueous solution failed to elicit a mutagenic effect in either of the *Salmonella* strains tested. However, it would seem that the selection of bacteria to test the mutagenicity of TCS is somewhat questionable, considering that TCS is a potent antimicrobial and would be highly toxic to the bacterial test species.

Currently available evidence from studies using classical assay systems indicates that TCS is neither genotoxic, mutagenic, or carcinogenic. However, there is some evidence that TCS is able to exert genotoxicity in non mammalian systems, including algae and bivalves. Due to the limited number of studies addressing the genotoxicity, mutagenicity, and carcinogenicity of TCS

in non- mammalian systems, it is difficult to conclude with certainty that the antimicrobial does not display a potential for harm.

#### *2.8.5 Reproductive and developmental effects*

The reproductive and developmental toxicity of TCS was assessed in several aquatic species, under controlled laboratory exposures. Orvos et al. (2002) investigated the early life-stage toxicity of TCS to rainbow trout. No statistical differences were observed in mean time to egg hatch among groups exposed to different concentrations of TCS in water, although swim-up behaviour was delayed in the 71.3  $\mu\text{g l}^{-1}$  treatment (Table 2.3). Decreased rates of fry survival were observed in this treatment group. Sublethal effects were also observed during the course of the study, and included a loss of equilibrium, locked jaw, erratic swimming, spinal deformities, and reduced activity.

The effects of TCS on early development and reproduction in medaka were studied by Ishibashi et al. (2004). In fertilized eggs exposed to 313  $\mu\text{g l}^{-1}$  of TCS, hatchability and time to hatching were significantly decreased and postponed, respectively. A 21 day exposure period to the antimicrobial failed to have any observable effect in the number of eggs produced and fertility, when comparing the control group to the 20, 100, 200  $\mu\text{g l}^{-1}$  TCS treatments. TCS appears to be quite toxic during the early life stages of medaka, and although the metabolite of TCS had weak estrogenic activity, the antimicrobial did not negatively impact the reproductive success of paired medaka or the survivability, growth, and sex ratios of the offspring.

In addition to the acute toxicity of TCS in adult fish, Oliveira et al. (2009) investigated the teratogenic effects of TCS on zebrafish larvae. The experiment was designed according to the OECD guideline on Fish Embryo Toxicity Test. Zebrafish embryos were exposed to five different concentrations of TCS; 0.1, 0.3, 0.5, 0.7 and 0.9  $\text{mg l}^{-1}$  for a 6 day period. The embryos were monitored daily for mortality, developmental parameters, and hatching. Additional larvae were collected for cholinesterase (ChE), glutathione S-transferase (GST), and lactate dehydrogenase (LDH) biomarker analyses. TCS exhibited acute toxicity for embryo/larvae (96 h  $\text{LC}_{50}$  of 0.42 mg

$l^{-1}$ ), resulting in delayed hatching, and mortality at 48 h. The teratogenic effects of TCS were observed at concentrations above  $0.7 \text{ mg } l^{-1}$ . The developmental effects of TCS included delayed otolith formation and eye and body pigmentation, spinal malformations, pericardial edema, and undersized larvae. In addition to embryo malformations, biomarker levels were also affected: ChE activity increased in the  $25 \text{ mg } l^{-1}$  treatment, GST activity increased in both the  $0.25$  and  $0.35 \text{ mg } l^{-1}$  treatments, and LDH activity increased in the  $0.25 \text{ mg } l^{-1}$  treatment. The results of this study indicate that TCS is toxic to zebrafish embryo/larva and negatively impacts hatching, embryonic development, enzyme activities, and survival. Based on the sensitivity of the biomarkers analyzed (GST, ChE, and LDH), the authors concluded that concentrations of TCS equal to or greater than  $0.3 \text{ mg } l^{-1}$  constitutes a significant environmental hazard.

Reproductive and teratological studies in rats, mice and rabbits carried out by Ciba-Geigy were reviewed by DaSalva et al. (1989), Bhargava and Leonard (1996), and Rodericks et al. (2010). In the rat study, TCS was administered in the diet. There were no effects on reproductive performance at any of the doses, including the highest dose of  $3000 \text{ mg } kg^{-1}$ . Effects in the offspring were detected only in pups from mothers fed the highest dose of TCS. In rabbits, TCS was administered by oral intubation to mothers, but no teratogenic effects were observed in the offspring. The reviews concluded that at doses of  $150 \text{ mg } kg^{-1}$  and higher, TCS is toxic to pregnant rats, but is not an overt teratogen. Similar conclusions regarding reproductive and developmental toxicity of TCS to mammalian species were reached by Rodericks et al. (2010). However, a study by Russell and Montgomery (1980), cited mostly for the failure to confirm mutagenicity of TCS in the mouse spot test, did provide some reproductive data for TCS. A single intraperitoneal dose of  $25 \text{ mg } kg^{-1}$  TCS affected the survival of embryos, significantly reducing litter size. In addition to reduced prenatal survival, an average dose of  $3.2 \text{ mg } kg^{-1}$  TCS resulted in significant decreases in postnatal survival. Despite the pronounced effects of TCS on survivability, very few externally identifiable abnormalities were observed in newborn mice in the higher dose groups. The study by Russell and Montgomery is one of the few studies to have

examined the effects of TCS on mammalian development, as more recent studies have focused primarily on aquatic species.

#### *2.8.6 Endocrine Disruption*

The structural similarity of TCS to known estrogenic and androgenic EDCs, including polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and bisphenol A, and to thyroid hormones (Norris and Carr, 2006; Veldhoen et al., 2006; Cabana et al., 2007; Allmyr et al., 2008) would be predictive, using the structure-activity relationship, of endocrine disruption. Several studies have shown that the antimicrobial has the ability to influence endocrine function in a variety of species (Table 2.4). This represents a considerable concern, as large amounts of TCS are used on a regular basis, with the antimicrobial having been detected in human plasma (Hovander et al., 2002), breast milk (Adolfsson-Erici et al., 2002), urine (Calafat et al., 2008), and the aquatic environment (Chalew and Halden, 2009).

##### *2.8.6.1 Thyroid Hormones*

Investigations of the propensity of TCS to disrupt thyroid homeostasis are based on the structural similarity of the antimicrobial to thyroid hormones. If TCS is indeed capable of perturbing the thyroid axis, the implications for developmental processes could be profound.

The first study to investigate the effects of polyhalogenated aromatic hydrocarbons (PHAHs), including TCS, on the metabolism of thyroid hormones was conducted, to the authors' knowledge, by Schuur et al. (1998). The *in vitro* inhibition of diiodothyronine (T<sub>2</sub>) sulfotransferase activity was measured using rat liver cytosol (Table 2.4). After an incubation of the PHAHs with induced liver microsomes, T<sub>2</sub> sulfotransferase inhibiting metabolites were formed. Specifically, the IC<sub>50</sub> for TCS was 3.1±0.7 µM. The results of this study were the first to indicate that TCS and its metabolites, like other hydroxylated halogenated compounds, are capable of inhibiting *in vitro* sulfation of thyroid hormones. Thyroid hormones are inactivated by sulfation, with sulfation playing a pivotal role in controlling thyroid metabolism during the developmental period (Norris and Carr, 2006). Schuur et al. (1998) postulate that fetal exposure

to hydroxylated compounds such as TCS, could result in the inhibition of thyroid hormone inactivation, with potential consequences to thyroid mediated developmental processes. Future studies are needed to address the in vivo potential of TCS to alter T2 sulfotransferase activity, and what, if any, effect that would have on the embryo and developing fetus.

To further assess the endocrine disrupting potential of TCS, Veldhoen et al. (2006) conducted a study to assess the potential of TCS to alter thyroid-mediated developmental processes in premetamorphic North American bullfrog (*Rana catesbeiana*) tadpoles (Table 2.4). Tadpoles were immersed in low concentrations of TCS for 4 days, and on day 4 were injected with 3,5,3'-triiodothyronine ( $T_3$ ) or a vehicle control. TCS exposure continued after the injection of  $T_3$ , ending on day 6, day 11, or day 18, with tissue samples collected and morphometric measurements made. Pretreatment with TCS concentrations as low as  $0.15 \mu\text{g l}^{-1}$  accelerated metamorphological changes following the administration of  $T_3$ . Within 48 h of  $T_3$  treatment,  $T_3$  mediated  $\text{TR}\beta$  mRNA expression in the tadpole tail decreased and levels of PCNA (proliferating nuclear cell antigen) transcript in the brain increased. In the absence of  $T_3$ , TCS alone affected thyroid hormone receptor  $\alpha$  transcript levels in the brain and resulted in transitory weight loss. The results of this study indicate that environmentally relevant levels of TCS are capable of disrupting developmental processes that are contingent on thyroid hormones in the bullfrog. Fort et al. (2010), exposed *Xenopus laevis* (South African clawed frog) larvae to TCS ( $0.6\text{-}32.3 \mu\text{g l}^{-1}$ ) over a 21-day exposure period. Although the authors concluded that TCS did not have an effect on larval development, thyroid histology, plasma thyroxine levels, and/or survivorship, the reported data suggest that TCS did have an effect on the postembryonic development of the tadpoles. Moreover, there was a significant difference between the exposed group and the control in the expression of  $\text{TR}\beta$ , which was induced by a magnitude of 1.5 in the  $1.5$  and  $7.2 \mu\text{g l}^{-1}$  TCS treatments. A reduction in larval growth in the  $1.5 \mu\text{g l}^{-1}$  treatment was also observed. The evidence available from amphibian studies suggests that metamorphosis of amphibians is highly sensitive to TCS and that the HQ value (PEC divided by NOAEC) may be greater than 1.0.

Following the study by Veldhoen et al. (2006), Crofton et al. (2007) tested the hypothesis that *in vivo*, TCS exposure influences serum levels of thyroxine (T<sub>4</sub>) in rats. Long-Evans females were given TCS (0, 10, 30, 100, 300, or 1000 mg kg<sup>-1</sup> day<sup>-1</sup>) via oral gavage for a short-term 4-day dosing schedule. Rats were sacrificed 24 h after the final TCS treatment and serum obtained. TCS doses of 100 mg kg<sup>-1</sup> day<sup>-1</sup> and higher decreased serum levels of T<sub>4</sub>, with the 30 mg kg<sup>-1</sup> day<sup>-1</sup> dose as the no observed effect level (NOEL). This study was the first to demonstrate that TCS decreases serum levels of T<sub>4</sub> in female rats. The effects of TCS on pubertal development and thyroid function in the male Wistar rat were investigated by Zorrilla et al. (2009). Prepubescent male rats were administered daily doses of 0, 3, 30, 100, 200, or 300 mg kg<sup>-1</sup> TCS via oral gavage for 31 days. At TCS doses of 30 to 300 mg kg<sup>-1</sup>, serum levels of T<sub>4</sub> decreased in a dose dependent fashion. However, observed decreases in serum levels of T<sub>4</sub> only corresponded to decreases in T<sub>3</sub> levels at the 200 mg kg<sup>-1</sup> dose, and colloid depletion was only observed in thyroid sections of the 300 mg kg<sup>-1</sup> treatment group. Compared to the controls, no significant differences in serum levels of thyroid-stimulating hormone (TSH) were noted in any of the treatments. To test the hypothesis that TCS decreases circulating T<sub>4</sub> levels by upregulating hepatic metabolism of thyroid hormones, Paul et al. (2010) used a 4-day exposure protocol in rats to analyze levels of hepatic enzyme induction (phase I and II enzymes), serum T<sub>4</sub>, serum T<sub>3</sub>, and TSH. Exposure to TCS caused a decrease in total T<sub>3</sub> and T<sub>4</sub>, an upregulation of mRNA expression and an increase in the activity of a number of phase I and phase II enzymes. The results of the study support earlier work, which has demonstrated that TCS-induced hypothyroxinemia is likely due to the induction of hepatic enzymes, which augment the catabolism of T<sub>4</sub>. Although it is possible that TCS may have direct effects on the thyroid gland and the production of thyroid hormones, previous studies have found no evidence to indicate this is occurring.

A study by Allmyr et al. (2009) is the first to have examined the effect of TCS on thyroid homeostasis in humans. Participants of the study brushed their teeth twice a day, for a period of 14 days, with a commercially available brand of dentifrice, Colgate total, containing 0.3% (w/w)

TCS. Blood samples were collected from the participants prior to TCS exposure and the day following the termination of the exposure period. Concentrations of TCS in plasma were significantly higher at the end of the exposure period. However, despite a significant difference between pre and post-exposure levels of plasma TCS, the increase in TCS had no effect on circulating levels of 4 $\beta$ -Hydroxycholesterol, a cholesterol metabolite used as an indicator of CYP3A4 activity, TSH, free T4, and free T3. The study concluded that the regular use of TCS containing toothpaste does not induce CYP3A4 activity or disrupt thyroid homeostasis. Unfortunately, the small study population coupled with the short-term exposure period, limit the validity of these findings. Future studies should aim to address the effects of long-term exposure to TCS in human subjects, from multiple exposure pathways, for prolonged periods of time.

Thus far, there has only been one human study (Allmyr et al., 2009) chronicling the effect of TCS on thyroid homeostasis. In this study, TCS did not alter circulating levels of thyroid hormones. In contrast, animal studies have shown that TCS decreases blood levels of T<sub>4</sub>, without concomitant changes in TSH concentrations. Environmentally relevant levels of TCS have also been shown to disrupt thyroid mediated developmental processes in premetamorphic North American bullfrog tadpoles (Veldhoen et al., 2006), though not in prometamorphic South African clawed frog tadpoles (Fort et al., 2010). TCS studies on thyroid disruption suggest that the antimicrobial is capable of acting on the thyroid receptor and altering the clearance of thyroid hormones, although future studies are needed to confirm this suspected mechanism of action and whether or not these effects are limited to animals. Given that TCS has been implicated in the disruption of thyroid hormone homeostasis and has been detected in breast milk samples of nursing mothers, the priority of future studies should be to ascertain whether or not TCS exposure can negatively affect fetal and postnatal development. A better understanding of the mechanisms of TCS-mediated thyroid disruption is warranted, in addition to species-specific differences.



#### 2.8.6.2 Sex Hormones

TCS is structurally similar to the anthropogenic estrogens, diethylstilbestrol and bisphenol A, in addition to the anti-estrogen, 2,3,7,8 tetrachloro-*p*-dibenzo-dioxin (TCDD) (Jacobs et al., 2005). Despite these structural similarities, an in vivo fish study by Foran et al. (2000) suggested that the antimicrobial was weakly androgenic, not estrogenic. In this study, Japanese medakas (*Oryzias latipes*) were exposed to TCS for 14 days, at concentrations of 1, 10, 100, 500  $\mu\text{g l}^{-1}$ , and 1  $\text{mg l}^{-1}$ . The effect of TCS on phenotypic sex ratios was determined by inspecting fin size and shape. The antimicrobial had no effect on the sex ratio of exposed fish, although a slight male bias in the 100  $\mu\text{g l}^{-1}$  treatment, and an accompanied difference in fin length between males of different exposure groups, indicated a possible anti-estrogen or a weakly androgenic effect (Table 2.4).

Ishibashi et al. (2004) further investigated the estrogenic potential of TCS. The estrogenic activity of TCS was measured using the induction of hepatic vitellogenin (Vtg) in male medaka, and an in vitro yeast two-hybrid assay. Hepatic Vtg levels were increased significantly in males exposed to TCS at 20 and 100  $\mu\text{g l}^{-1}$ , although this was not the case in the 200  $\mu\text{g l}^{-1}$  treatment group. The estrogenic activity of TCS was measured in the yeast two-hybrid assay alone and in the presence of rat S9 liver fractions. Alone, TCS had a weak estrogenic activity, but with the addition of the rat S9 liver treatment, the estrogenic activity of the antimicrobial was increased two-fold. The results of the study suggest that the metabolite of TCS is a weak estrogen, with the potential of inducing Vtg in male medaka.

Houtman et al. (2004) identified TCS at relatively high concentrations in the bile of male breams (*Abramis brama*) collected at river sites in the Netherlands. The estrogenic potencies of TCS and other compounds in the bile were assessed using the ER-CALUX (Estrogen Responsive Chemical Activated Luciferase Gene Expression) assay (Houtman et al., 2007). Estradiol and estrone were the major contributors to estrogenic activity, where TCS concentrations of up to 0.1mM gave no indication of any estrogenic activity. The authors concluded that the

antimicrobial did not contribute significantly to the estrogenic activity measured in the bile of male breams. It is important to keep in mind that key differences between the toxicity of compounds in vivo and in vitro exist.

The potential of TCS to induce Vtg production and decrease sperm counts, both being well established biomarkers of endocrine disruption, was assessed in male mosquito fish (*Gambusia affinis*) by Raut and Angus (2010). A 35 day exposure period to TCS induced Vtg production and decreased sperm production, moreover, the hepatosomatic index of TCS exposed fish was significantly elevated compared to controls. However, it is important to note that in this study, endocrine disruption was observed at TCS concentrations approximately 100 times greater than those typically detected in surface water, and as such, it is not known if environmentally relevant concentrations of TCS would produce similar results.

Matsumara et al. (2005) investigated the effects of TCS on plasma Vtg levels, testosterone synthesis, and hepatic CYP1A and CYP2B activities in male *Xenopus laevis*. Waterborne TCS at environmentally relevant concentrations did not have any estrogenic effects, while male frogs treated with intraperitoneal injections of TCS at 4-400  $\mu\text{g g}^{-1}$  body weight had lower plasma Vtg and testosterone levels than the control group. Hepatic CYP1A and CYP2B activity, as measured by ethoxyresorufin O-deethylase (EROD) or 7-pentoxoresorufin O-depentylase (PROD), was not significantly different from the controls. The authors hypothesized that the observed decrease in plasma Vtg may be partially explained by the (anti)estrogenic effects of TCS in male *X. laevis*.

The evidence provided by fish and amphibian models suggests that TCS has endocrine disrupting activity, however the number of studies with these species is limited. The research efforts to assess endocrine disruption in mammalian models are more extensive. A potential explanation for TCS's ability to act as an endocrine disruptor comes from evidence that the antimicrobial activates the human pregnane X receptor (hPXR). This receptor is stimulated by a wide array of environmental chemicals and is responsible for inducing enzymes that metabolise

steroids and detoxify xenobiotics (Jacobs et al., 2005). In the study by Jacobs et al. (2005), the human hepatoma cell line (HuH7) was used to quantify PXR activity. Cells were exposed to concentrations of TCS from 0.01  $\mu\text{M}$ -10  $\mu\text{M}$  and the capacity of TCS to induce PXR activity was expressed as the percentage of the positive control. At 46.2%, TCS proved to be a moderate inducer of hPXR activity. In contrast to the other compounds tested, TCS was the only one to show concomitant increases in % max induction, with doses above 10  $\mu\text{M}$ .

Subsequently, Chen et al. (2007) tested the in vitro (anti)androgenic effect of TCS on testosterone induced transcriptional activity, in a cell line lacking essential steroid metabolizing enzymes. These cells (2933Y) are highly sensitive to endogenous steroids, in addition to anthropogenically sourced endocrine disruptors. At TCS concentrations of 1.0  $\mu\text{M}$  and 10  $\mu\text{M}$ , testosterone-induced transcriptional activity was reduced by 38.8% and 92%, respectively. In the absence of testosterone, TCS did not exhibit any androgenic activity, at concentrations up to 10  $\mu\text{M}$ . A second study to test the in vitro endocrine disrupting effects of TCS was done by Gee et al. (2008). This study examined both the estrogenic and androgenic activity of TCS in

**Table 2.4:** Endocrine-disrupting effects of Triclosan (TCS)

Test species/system	Life stage	Aquatic system	Route of exposure	Test duration	TCS exposure	Effects	Reference
<b>Fish</b>							
Medaka ( <i>Oryzias latipes</i> )	embryos	FW	Water	14 d	100 µg l <sup>-1</sup>	Weak androgenic (or anti-estrogenic) effect (↑male fin size, slight male bias sex ratio)	Foran et al. 2000
	Male fish	FW	Water	14 d	20 µg l <sup>-1</sup>	Weak estrogenic activity; ↑Vtg in male fish; activity in yeast assay	Ishibashi et al. 2004
Mosquitofish, ( <i>Gambusia affinis</i> )	Male fish	FW	Water	35 d	101.3 µg l <sup>-1</sup>	↑vitellogenin, ↓ sperm count	Raut and Angus, 2010
Bream ( <i>Abramis brama</i> )	Bile of male fish	FW	Field sites Netherlands		No activity up to 0.1mM	No estrogenic activity detected in ER-CALUX assay	Houtman et al. 2004
<b>Amphibians</b>							
North American bullfrog ( <i>Rana catesbeiana</i> )	tadpoles	FW	In vivo	18 d	0.15 µg l <sup>-1</sup>	Disruption of T3-dependent developmental metamorphosis processes	Veldhoen et al. 2006
South African clawed frog ( <i>Xenopus laevis</i> )	tadpoles	FW	In vivo	21 d	1.5 µg l <sup>-1</sup> 0.6-32.4 µg l <sup>-1</sup>	↓larval growth; No effect on metamorphosis	Fort et al. 2010
	males	FW	Water; ip injection	14 d	20-200 µg l <sup>-1</sup> ; inject 4-400 µg g <sup>-1</sup>	No effect on Vtg in males; no effects on	Matsumara et al. 2005

				body weight	CYP1A and EROD; ↓ Vtg in ip injected males	
<b>Mammals</b>						
Sheep	placenta	In vitro		0.6 nM	↓estrogen sulfonation (IC <sub>50</sub> )	James et al. 2009
Rats (Wistar)	Pre-pubescent males	Oral (gavage)	31 d	200 mg kg <sup>-1</sup>	No effect on timing of puberty; ↓levels of plasma testosterone and T4	Zorrilla et al. 2009
				30 mg kg <sup>-1</sup>		
	Males; Isolated Leydig cells	In vivo (daily intubation) In vitro	60 d	5-20 mg kg <sup>-1</sup>	Disruption of LH, FSH, pregnenolone and testosterone synthesis; ↓mRNA expression of StAR and steroidogenic enzymes	Kumar et al. 2008; 2009
			2 h	0.01-10 μM		
Rats (Long Evans)	Adult female	Oral (gavage)	4 d	100 mg kg <sup>-1</sup> day <sup>-1</sup>	↓plasma T4	Crofton et al. 2007
	Females Weanlings	Oral (gavage)	4 d	300 mg kg <sup>-1</sup> day <sup>-1</sup>	↓plasma T4 and T3	Paul et al. 2010
Human	adults	brush 2/day with TCS tootpaste	14 d	0.3%w/w TCS	No effect on thyroid status	Allmyr et al. 2009
<b>Cell-based assays</b>						
MCF37 breast cancer cells		In vitro		10 μM	Estrogenic and androgenic effects	Gee et al. 2008

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2933Y cells (human)	In vitro	1.0 $\mu$ M and 10 $\mu$ M	$\downarrow$ testosterone-induced transcriptional activity	Chen et al. 2007
Cell-based nuclear-receptor-responsive and calcium signaling bioassays (AhR, ER, AR, RyR)	In vitro	1-10 $\mu$ M (for ER- and AR-responsive gene expression; 0.1-10 $\mu$ M (for RyR response)	Weak AhR activity; antagonistic activity in ER- and AR-dependent gene expression; interaction with RyR1, $\uparrow$ Ca <sup>2+</sup> mobilization in skeletal myotubes	Ahn et al. 2008
HuH7 cells (human hepatoma cell line) transfected with human pregnane X receptor (hPXR)	In vitro	>10 $\mu$ M	Activation of hPXR	Jacobs et al. 2005
Induced rat liver microsomes	In vitro	3.1 $\mu$ M (IC <sub>50</sub> )	$\downarrow$ diiodothyronine (T <sub>2</sub> ) sulfotransferase activity	Schuur et al. 1998

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breast cancer cells. At environmentally relevant concentrations, TCS was capable of producing both estrogenic and androgenic effects. TCS displaced radiolabelled estradiol from estrogen receptors of MCF7 human breast cancer cells, whilst also inhibiting testosterone from binding to the rat androgen receptor.

The endocrine disrupting potential of TCS was further investigated by Ahn et al. (2008) using in vitro cell-based and nuclear-receptor-responsive bioassays for aryl hydrocarbon (AhR), estrogen (ER), androgen (AR) and ryanodine receptors. The results of the cell-based AhR-mediated bioassay demonstrated that TCS is an AhR inducer, a receptor which has been implicated in various toxic and biological responses. In both the cell-based ER- and AR-mediated bioassays, TCS acted antagonistically, but was a powerful disruptor of Ca<sup>2+</sup> regulation. The authors concluded that the results of their study provided sufficient reason to be concerned about the antimicrobial's neurotoxic potential. These results are further supported by previous studies that have shown that TCS alters thyroid homeostasis (Veldhoen et al., 2006). Moreover, there is evidence that TCS can also influence endocrine function indirectly, through effects on the metabolism of key hormones, including the thyroid hormones. The effects of TCS on EROD, PROD, UDP-GT and sulfotransferase enzymes, all of which play a role in the metabolism and clearance of hormones from the body, have been reported (Hanoika et al., 1997; Jinno et al., 1997; Kanetoshi et al., 1992; Schuur et al., 1998). In addition, several in vivo studies have shown that TCS has endocrine disrupting effects.

Further evidence for the (anti)androgenic effect of TCS was provided by Kumar and colleagues, who sought to describe the targets of TCS endocrine disruption, in addition to the mechanism(s) of action. An earlier study with Leydig cells exposed to TCS in vitro (Kumar et al., 2008), was followed by a whole animal study (Kumar et al., 2009). Male rats were dosed with 5, 10, or 20 mg kg<sup>-1</sup> of TCS per kg of body weight per day. Rats were treated with TCS once a day for a period of 60 days. RT-PCR analysis indicated that TCS decreased mRNA levels for testicular steroidogenic acute regulatory (StAR) protein, cytochrome P450<sub>sc</sub>, cytochrome

P450<sub>C17</sub>, 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD), 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD), and androgen receptor (AR). The translation of testicular StAR and AR protein was also disrupted by the antimicrobial. Decreases in serum levels of lutenizing hormone (LH), follicle stimulating hormone (FSH), cholesterol, pregnenolone, and testosterone were observed. Histopathological analysis of the testes and sex accessory glands were indicative of widespread malformations. TCS-induced decreases in testosterone and spermatogenesis were likely the result of decreases in serum levels of LH and FSH, thereby implicating the pituitary-gonadal axis, at various levels, as a target for endocrine disruption.

The reproductive effects of TCS on testosterone-dependent endpoints in rats were not as clearly evident in a study by Zorrilla et al. (2009), which sought to assess the effect of TCS on puberty, as well as thyroid hormones (see section above) in male Wistar rats. The antimicrobial had no effect on the growth or onset of preputial separation. A significant difference in serum testosterone levels was observed in the 200 mg kg<sup>-1</sup> treatment, but not in the 300 mg kg<sup>-1</sup> treatment. In addition, the age of pubertal onset and the development of androgen-dependent reproductive tissues did not differ significantly between the experimental and control groups.

James et al. (2009) tested the vulnerability of the placenta to the endocrine disrupting effects of TCS. TCS proved to be a powerful inhibitor of estradiol sulfonation in the placental tissue of sheep, with IC<sub>50</sub> of 0.6 nM TCS. As the majority of estrogen secreted by the placenta is sulfoconjugated and estrogen sulfonation have been linked to pregnancy loss (Tong et al., 2005), TCS could potentially have a negative impact on the fetal environment and pregnancy maintenance. Environmental and/or household exposure to TCS in humans can lead to blood levels in the low nM range (Allmyr et al., 2006, 2008) The possibility then exists for placental levels of TCS to reach concentrations high enough to interfere with placental estrogen metabolism.

There is good evidence for the endocrine disrupting effects of TCS, although it remains unclear as to whether TCS has (anti)estrogenic effects, (anti)androgenic effects, or both. Foran et



al. (2000) concluded that based on changes in fin length and slightly skewed sex ratios in medaka fish, TCS is a weak androgen. A later study by Ishibashi et al. (2004), reported that a TCS metabolite induced Vtg production in male medaka, suggesting estrogenic activity. Evidence from amphibian studies supported the role of TCS as an anti-estrogenic chemical. Several in vitro studies have demonstrated the potential for TCS to act as an anti-estrogen and/or anti-androgen (Chen et al., 2007; Gee et al., 2008; Ahn et al., 2008). Studies with male rats (Zorrilla et al., 2009; Kumar et al., 2009) have shown that TCS decreases serum levels of testosterone and the activity of several important steroidogenic enzymes. Lastly, TCS is a powerful inhibitor of estrogen sulfonation in sheep placental tissue, and as such, could have deleterious effects on the ability of female mammals to maintain a full term pregnancy. Because TCS has been shown to have estrogenic and androgenic activity at environmentally relevant levels, there is sufficient reason to be concerned about the impact of the antimicrobial on aquatic ecosystems and human health.

## **2.9 Efficacy and Antibacterial Resistance**

### *2.9.1 Efficacy*

The efficacy of TCS-containing consumer products has recently been called into question, as several studies have reported that the antimicrobial is no more effective than regular soap.

#### 2.9.1.1 Health-care settings

The efficacy and safety of TCS in health care settings was reviewed by Jones et al. (2000). The popularity of TCS in health care settings has long endured the test of time, as the antimicrobial has proven its immediate, broad-spectrum antimicrobial effects, in addition to the fact that TCS elicits neither dermal irritation nor photosensitization effects. Interestingly, cell culture experiments have even shown that the antimicrobial has anti-inflammatory, anti-allergic, anti-asthmatic activity and can even protect against cell damage. All of the aforementioned characteristics are very important for ensuring antimicrobial acceptance and compliance in health care settings.

The antimicrobial effects of TCS have been established for concentrations of 0.2 to 2.0%. TCS formulations of 1% have been proven effective for managing antibiotic-resistant *Staphylococcus aureus* outbreaks in health care settings, in the form of either hand wash solutions or bathing antiseptics (Jones et al., 2000). TCS is often favoured over other antimicrobial products due to its mild nature, helping to increase handwashing compliance in health care workers. According to the authors, a 1% TCS formulation is preferable for securing high rates of hand washing compliance and reducing nosocomial infections in high-risk, high-frequency handwashing health care settings (see Jones et al., 2000). It is worth reinforcing that while the use of TCS formulations appears to be beneficial in high-risk, high-frequency handwashing health care settings, these benefits do not necessarily translate into the domestic domain.

#### 2.9.1.2 Personal Care Products

As previously mentioned, a staggering number of personal care products contain TCS, at varying concentrations. As TCS is not subject to stringent government regulation, concentrations of TCS in consumer products can vary substantially, although they generally remain in the range of 0.1-0.45% (w/v) (Aiello et al., 2007). A review on the efficacy of TCS containing hand soaps, with efficacy defined as antibacterial activity above and beyond that of plain hand soap, revealed that at concentrations typically present in antibacterial soaps, TCS was not superior for reducing bacterial counts on the hands or decreasing the prevalence of infectious diseases (Aiello et al., 2007). In studies that showed TCS reduced bacterial populations on the hands (Bhargava and Leonard, 1996), longer hand washes and/or high concentrations of the antimicrobial were used, accounting for the higher efficacies recorded. Due to the lack of data supporting the efficacy of TCS containing antimicrobial soaps, the use of these products seems unnecessary in light of concerns about the potential for the selection of antibiotic cross-resistance and impacts on the aquatic environment.

Despite a lack of data on the efficacy of TCS in antibacterial soaps, there is a growing body of evidence to suggest that in oral care formulations, TCS and TCS/copolymers deliver

effective antibacterial protection by causing bacterial lysis. TCS is the most commonly used antibacterial agent in oral care formulations (van den Broek et al., 2008). According to a review on the management of halitosis by van den Broek et al. (2008), a dentifrice formulated with 0.3% TCS, 2.0% of a copolymer of polyvinyl methyl ether maleic acid, and 0.243% sodium fluoride significantly reduced the incidence of organoleptic sores and hydrogen sulphide releasing bacteria. Moreover, the antimicrobial and anti-inflammatory properties of TCS have been proven to reduce plaque and gingivitis, in addition to slowing the progression of periodontal disease (Rosling et al., 1997; Cullinan et al., 2003; Gunsolley, 2006; Rautemaa et al., 2007). The long-term use of antimicrobials is however worrisome, as resistance is always a lingering concern, although there is no data to suggest that TCS-resistant strains of pathogens have emerged alongside the incorporation of the antimicrobial into oral care formulations (Rosling et al., 1997; Cullinan et al., 2003; Rautemaa et al., 2007). Based on the evidence from several review studies, the weighing of the risks and benefits of TCS in oral care formulations strongly favours the use of the antimicrobial in dentifrice and mouthwashes (Sreenivasan and Gaffar, 2002; Gilbert et al., 2007).

#### 2.9.1.3 Plastics and other materials

The number of applications for TCS has expanded immensely over the years, with the antimicrobial now being impregnated in a number of different materials, ranging from medical devices to athletic clothing to meat packaging, in the hopes of providing the user with long-lasting antibacterial protection. The extent to which TCS is able to prevent the proliferation of bacterial populations in many of these products has not been adequately established. Ensuring that TCS is consistently released, at concentrations that are high enough to limit bacterial growth, remains a significant challenge for manufacturers.

The use of TCS in textiles has been banned in Europe, due to concerns of antibiotic resistance and the generation of toxic by-products, primarily 2,8-dichlorodibenzo-*p*-dioxin. However, manufacturers in North America continue this practice on a wide scale basis. The

premise of this manufacturing process is that TCS will migrate to the surface of the textile, providing extended antimicrobial release throughout the lifespan of the fibre (Gao and Cranston, 2008). Despite these claims of antibacterial protection, experimental evidence to support the practice of impregnating clothing fibres with TCS remains limited.

There is no doubt that manufacturers are feeling increased pressure from consumers to create products that provide extended antibacterial protection. The development of Microban<sup>®</sup> products, an innovative technology that enables TCS to be incorporated into virtually any type of plastic material (either directly into the plastic or as a coating with a second type of film), while allowing the antimicrobial to accumulate on the surface to inhibit bacterial growth, has garnered the attention of the food packaging industry (Vermeiren et al., 2002; Quintavalla and Vicini, 2002). It has been thought that by incorporating antimicrobial agents such as TCS, into food packaging, the shelf-life and safety of the product(s) would be ameliorated. Although this technology seems very promising, several in vitro studies demonstrated that TCS incorporated products do little to prevent the growth of bacteria in commercial applications, likely due interactions between TCS and food particles (Cutter, 1999; Vermeiren et al., 2002).

The effectiveness of TCS incorporated plastic has been investigated by Cutter (1999). In plate overlay assays, TCS demonstrated antibacterial activity, but when the plastic was vacuum-packaged and refrigerated, bacterial growth was not sufficiently reduced. The authors attributed the failure of the TCS polymer to inhibit bacterial growth to the interactions between TCS and adipose deposits in meat. The antimicrobial efficacy of a polymer coated in TCS, for the purpose of packaging perishable foods, has also been evaluated in a study by Chung et al. (2003). The applicability of the study is limited, in that only one type of bacteria was used, *Enterococcus faecalis*, in addition to the fact that incubations were carried out at one temperature only, 30°C. The results of the study specify that only minute amounts of the antimicrobial are released from the polymer, although one must consider that the minimum inhibitory concentration for TCS is fairly low. Despite only small amounts of TCS being released, bacterial inhibition by the TCS

coating was clearly seen in both the agar diffusion test and a liquid culture test, although neither test was performed at refrigeration temperature. Plate overlay assays at refrigeration temperature, with different bacterial strains, are needed to confirm the antimicrobial efficacy of TCS in food packaging.

More recently, Camilloto et al. (2009) evaluated the antimicrobial efficacy of three different concentrations (0, 2000, and 4000 mg kg<sup>-1</sup>) of a TCS active film in the preservation of sliced ham. The activity of the film was tested both in vitro and in sliced ham inoculated with *E.coli* and *S. aureus*. In the in vitro experiment, film efficiency was measured by the diameter of the inhibition halos around the TCS containing film, in agar plates that had been inoculated with *E. coli*, *S. aureus*, *L. innocua*, *S. choleraesius*, or *P. aereginosa*. The film only showed inhibition effects against *E.coli* and *S. Aureus*. Correspondingly, in the sliced ham packaged with the TCS containing film, a reduction of 1.5 logarithmic cycles for *E.coli* and *S. aureus* was observed after 12 days of storage at 7±2

□C. The effica

suggestive of a potential for the use of the antimicrobial in food packaging, as the film controlled the proliferation of certain types of bacteria that cause food borne illnesses.

Studies on the efficacy of TCS incorporated film have been far from conclusive, perhaps in part due to methodological variations. The efficacy of these films is likely dependent on the type of polymer material, the method of TCS incorporation and production, the concentration of antimicrobial used, and also the bacterial strains and conditions used (Camilloto et al., 2009). Testing antimicrobial films at refrigeration temperatures, with a variety of meat products, is paramount, since this type of packaging is generally used with refrigerated foods and fatty acids from meat are thought to decrease the efficacy of TCS containing films (Cutter, 1999).

#### 2.9.1.4 Medical Devices

Medical devices are often a site of bacterial proliferation, which can cause debilitating infections and even death. Bacterial adhesion to medical devices is a serious medical problem that places a significant strain on the health care system. A proposed solution to this problem has been

the incorporation of antibacterial agents into medical polymers. The antibacterial potency of TCS incorporated into plasma-modified medical polyethylene (PE) and bulk PVC has been investigated (Zhang et al., 2006; Ji and Zhang, 2008). The results of these two studies demonstrated that TCS-incorporated bulk PVC significantly reduced bio-film on polymer surfaces and that plasma-modified PE with TCS provided adequate antibacterial protection. However, these studies were laboratory based, and it has been previously shown in antibiotic cross-resistance studies that the effects observed in the laboratory setting often do not translate to the 'real world'. Further studies are needed to confirm that TCS impregnated medical polymers are genuinely able to reduce bacterial adhesion and proliferation.

The results of the studies by Zhang et al. (2006) and Ji and Zhang (2008) do not agree with previous studies on the antibacterial efficacy of TCS incorporated polymers. Junker and Hay (2004) compared biofilm populations on ABS plastic impregnated with or without TCS, after 1-3 weeks of exposure to drinking water. A lack of measurable differences in bacterial populations between TCS-impregnated and control plastic was observed, a phenomenon which can be explained by the fact that only a minute amount of TCS actually migrated from the plastic.

Previously, Kalyon and Olgub (2001) investigated the antibacterial efficacy of TCS-incorporated polymers, reporting that TCS was only capable of inhibiting bacterial growth for a limited period of time, after which bacterial growth flourished. The authors suggested that the majority of TCS was not available to bacteria, as was evidenced by the fact that the amount of TCS incorporated into the polymer was much higher than the minimum inhibitory concentration (MIC) for the bacterial species. The findings of this study are in concordance with those of Imazato et al. (1995), who studied the efficacy of dental polymers containing TCS. The TCS incorporated polymer composites reduced bacterial proliferation for 12 hours, but once the 24 hour mark had been reached, the number of bacteria on the surface of the control and the TCS-incorporated composite were virtually the same.

Despite the fact that TCS impregnated polymers are commercially available in a wide range of products, few studies have been able to document their efficacy. With the noteworthy exception of oral care products, the antimicrobial efficacy of numerous TCS-containing products has yet to be validated. There is an urgent need to critically evaluate the false sense of security that the mass marketing of antimicrobial products creates, considering that the injudicious use of antimicrobials may lead to antibiotic cross-resistance and should be avoided.

### *2.9.2 Antibiotic cross-resistance*

Traditionally, TCS was thought not to be implicated in antimicrobial resistance because of its broad-spectrum antibacterial properties and multiple bacterial targets. However, this understanding has been called into question, as several studies have demonstrated the potential for TCS to target a specific bacterial enzyme, enoyl-acyl carrier protein reductase (McMurray et al., 1998). Indeed, laboratory studies have shown that TCS-resistant bacteria can be cultured with relative ease, and it has been suggested that TCS-resistant bacteria may be the result of mutations in and/or the over-production of enoyl reductase, changes in the membrane permeability, or efflux (Russel, 2004). However, it is important to remember that at higher concentrations, biocides, like TCS, have widespread targets. It is usually only at lower concentrations, not typically in-use concentrations, that biocides become more selective in their targets (Russell, 2003, 2004).

The fact that TCS can target a specific enzyme could potentially be a public health issue in the future, as antimicrobials are not intended to target any particular cellular constituents in bacteria (Jones et al., 2000). Instead, it is antibiotics that exert their destructive powers by targeting specific cellular components of bacteria. Unfortunately, it is the case that these cellular targets regularly undergo mutations, subsequently rendering the antibiotic ineffective. Once it was discovered that TCS had the ability to target a specific enzyme in bacteria, researchers began speculating that the antimicrobial may have the potential to prime bacteria for antibiotic cross-resistance.

A review by Russell (2004) examined the link between TCS and antibiotic resistance. Typical in-use concentrations of TCS generally far exceed minimal inhibitory concentrations (MICs) for most bacterial species, with the exception of *P. aeruginosa*. A TCS-susceptible mutant of *P. aeruginosa*, when exposed to TCS, activates an efflux pump that decreases the susceptibility of the bacterial strain to ciprofloxacin (Russell, 2003). However, later studies have failed to implicate TCS in ciprofloxacin resistance in the clinical setting. Nor is there any evidence to suggest that TCS is linked to antibiotic resistance in *S. aureus*. Moreover, comprehensive surveys on the use of TCS in the home have failed to correlate the antimicrobial with antibiotic resistance (Cole et al., 2003).

A later review by Yazdankhah et al. (2006) concluded that although cross-resistance between TCS and other clinically relevant antimicrobials has been documented for *E. coli* and *Salmonella* spp. in the laboratory setting, these findings have yet to be confirmed in clinical environments. However, the authors of the study draw attention to the fact that very few studies have been done on clinical isolates, suggesting that future studies on this topic may yet expose such a link. There is also a large knowledge gap in the literature concerning the impact of TCS use on commensal bacteria, which have the potential to transfer resistance to bacterial strains that are known to be human pathogens. Future studies should address the effect of TCS on bacteria under typical conditions in community and health care settings, and the relationship between TCS use and microflora.

Interestingly, some researchers have suggested that although TCS resistance has been associated with antibiotic cross-resistance in the laboratory setting, these findings do not carry over to clinical environments (Russell 2003, 2004; Gilbert et al., 2007; Aiello et al., 2007). This discrepancy is not necessarily surprising, as laboratory tests on TCS resistance have used predominantly pure cultures, in nutrient rich environments, both of which are not representative of real world conditions (Gilbert et al., 2007). As it currently stands, there is a lack of clinical evidence to suggest that the use of TCS has led to the propagation of antibiotic resistant



staphylococci, antibiotic-resistant Gram-negative bacteria, or isoniazid-resistant *M. tuberculosis*, although some researchers continue to speculate that this type of cross-resistance is just over the horizon (Russell, 2003; Aiello et al., 2007).

Conclusions about TCS and a lack of antibiotic cross-resistance in a variety of environments must be drawn with a considerable degree of trepidation, as studies examining the issue have not provided enough evidence to assuage fears that the use of the antimicrobial is devoid of risk. Based on the fact that antibiotic cross-resistance has been demonstrated in the laboratory setting, further studies are needed to confirm that in clinical, household, and community settings, the judicious use of TCS does not, and will not, lead to antibiotic resistance. It would then seem logical to suggest that gratuitous use of TCS should be all but eliminated, whilst allowing for the continuation of its important clinical functions. The use of TCS should be limited to applications where it has been demonstrated to be effective, which includes health care settings and in oral hygiene formulations (Gilbert and McBain, 2002; Cozad and Jones, 2003; Russell, 2004). Currently, the Canadian Medical Association is calling for a ban on antibacterial products because of concerns that these products may actually promote bacterial growth. Previously, many European countries, such as Sweden, have actively discouraged consumers from using antimicrobial products.

## **2.10 Conclusions and Further Research**

Based on the existing literature, the judicious use of TCS should be considered safe, and although antibacterial cross-resistance has been found in the laboratory setting, there is no evidence to suggest that it is occurring in clinical and/or household environments. However, there are a number of different issues surrounding the use of TCS that warrant further research; environmental by-products, bioaccumulation potential, toxicity to aquatic organisms, endocrine disrupting effects, and the potential for TCS to prime bacteria for antibiotic resistance. These issues are of importance for the safeguarding of human health, aquatic ecosystems, and the environment.

**Acknowledgements** – We would like to thank the anonymous reviewers, especially reviewer #3, for their critical comments and suggestions about this review.

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**CHAPTER 3. PRIMARY RAINBOW TROUT ADRENOCORTICAL CELL CULTURE –  
MODIFICATION OF THE METHOD ORIGINALLY DEVELOPED BY  
LEBLOND AND HONTELA (1999)**

**3.1 Introduction**

A number of different chemicals are commonly detected in surface water (Buchanan et al., 2010; Wu et al., 2010), yet the effects of these complex chemical mixtures on aquatic species remain relatively unknown. Chemicals can interact with other compounds in the water to increase or decrease their toxicity, potentially jeopardizing the health and survival of aquatic organisms. The use of fish species in biological research has been increasing steadily since the 1960's, as researchers are beginning to recognize the advantages of choosing to work with fish models, instead of more traditional mammalian systems (Bolis et al., 2001). *In vitro* systems, including fish cell cultures, make excellent models for environmental toxicology research (Bolis et al., 2001).

A primary cell culture of the rainbow trout head kidney where cortisol producing adrenocortical cells are located, has been developed by Leblond and Hontela (1999) and will be henceforth referred to as the adrenocortical bioassay. The adrenocortical bioassay is well-suited for toxicological studies, as a biochemical tool for monitoring the effects of water-borne chemicals on corticosteroid synthesis. A significant advantage of the Leblond and Hontela primary cell culture, over the human adrenocortical tumour cell line (H295R), is that the H295R cell line does not adequately respond to ACTH stimulation, due to low levels of ACTH receptor expression (Masters and Palsson, 1999). It then follows, that studies using the ACTH challenge test would need to select a primary cell culture method of adrenal cells instead.

In fish, corticosteroids play an important role in modulating metabolism, osmotic regulation, immune function, and reproduction (Mommsen et al., 1999). The head kidney of teleost fish is similar to the adrenal gland of mammals; the head kidney produces cortisol in response to ACTH, although the cortisol producing cells do not form a compact gland (Mommsen

et al., 1999). The ACTH challenge test (Hontela and Vijayan, 2009) can be used to screen for endocrine disrupting effects of water-borne toxicants using rainbow trout head kidney cells in a primary culture and a cortisol radioimmunoassay. Typically, results from *in vitro* toxicological studies are confirmed with *in vivo* experiments and a mechanism of action elucidated.

A limitation of the Leblond and Hontela method is the considerable amount of cell aggregation that typically occurs following tissue digestion. In order to address this challenge and other less significant problems, such as low cortisol secretion and subsequent high cell densities required for plating, the method was modified. The objective of the current study was to compare the performance of the modified primary culture method to the original method (Leblond and Hontela, 1999), using qualitative and quantitative comparisons, including the degree of cell dispersion, cell yield, and the amount cortisol secreted in response to the ACTH challenge test.

### **3.2 Materials and Methods**

#### *3.2.1. General*

Male and female juvenile rainbow trout (*Oncorhynchus mykiss*), body weight  $58.8 \pm 3.3$ g, were obtained from the Allison Creek Brood Trout Station (Crowsnest Pass, Alberta, Canada). Fish were maintained in a 1500-L flow-through tank at 13-15<sup>0</sup>C, on a photoperiod of 12L: 12D. Fish were fed to satiation with commercial trout chow food. Prior to the commencement of tissue harvesting, fish were acclimated in the laboratory for a period of two weeks. Tissue culture media, digestion enzymes, ACTH, bovine serum albumin, and sodium bicarbonate, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Cortisol radioimmunoassay kits were purchased from MP Biomedicals (#07-221102, Medicorp, Montréal, Canada).

#### *3.2.2. Preparation of adrenocortical cell suspensions (Leblond and Hontela, 1999; original method)*

Fish were anesthetized with MS-222 (0.5g l<sup>-1</sup> of water) buffered with sodium bicarbonate to a pH of 7. A 1-cc heparinized syringe was used to remove a maximum blood sample from the

caudal vein. Following blood sampling, fish were perfused through the heart with 30 ml of a 0.7% saline solution. The head kidney was excised and the tissue placed in room temperature MEM. Tissue fragments were minced into 1-mm<sup>3</sup> and transferred into a 15 ml conical centrifuge tube for enzymatic dispersion using collagenase/dispase and DNase, as previously described (Leblond and Hontela, 1999). Tissue was digested in a shaker incubator for one hour at 23<sup>0</sup>C and gently pipetted every 15 min to further facilitate cell dispersion.

Digested tissue was filtered through nylon mesh (30µM pore size). Undigested tissue was mechanically dispersed with a plastic transfer pipet and the cell suspension filtered. The cell suspension was centrifuged for 5 min at 200g. The pellet was re-suspended in 0.75 mL of MEM and the cell yield and viability determined by counting the cells in a hemocytometer in the presence of trypan blue. The cell suspension was adjusted to a cell density of 75 X 10<sup>6</sup> and plated at a volume of 100 µL per well in a 96-well microplate. Following plating, cells were incubated in MEM at 15<sup>0</sup>C for two hours prior to ACTH stimulation.

### 3.2.3 *Stimulation of cortisol secretion*

After centrifugation, cells were re-suspended in 100 µL of MEM containing 1 U ml<sup>-1</sup> ACTH (Porcine adrenocorticotropin, ACTH 1-39, Sigma). Cells were incubated in an environmental chamber for 1 hour at 15<sup>0</sup>C. Following the one-hour stimulation, cells were centrifuged for 5 min at 200g and the supernatant collected and frozen at -80<sup>0</sup>C. Cortisol secretion (ng ml<sup>-1</sup>) in the supernatant was assessed using a radioimmunoassay for cortisol (MP Biomedicals).

### 3.2.4 *Method modifications*

Fish were placed on ice and a 1-cc heparinized syringe was used to remove a maximum blood sample from the caudal vein. The head kidney was excised and the tissue placed in ice cold medium, 0.02% Ethylenediaminetetraacetic acid disodium salt (EDTA) prepared in Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca<sup>2+</sup> and Mg<sup>2+</sup>. Prior to enzymatic digestion, tissue fragments were rinsed with ice cold 0.02% EDTA and minced into 1-mm<sup>3</sup> pieces with two

scalpels. Tissue was digested in a shaker incubator for two hours at 21<sup>0</sup>C. Digested tissue was washed with 0.02% EDTA and filtered through nylon mesh (30 $\mu$ M pore size). The pellet was re-suspended in 1 mL of 0.02% EDTA and the cell yield and viability determined by counting the cells in a hemocytometer in the presence of trypan blue. Following plating, cells were centrifuged for 5 min at 200g and re-suspended in MEM for a two hour incubation period prior to ACTH stimulation.

### 3.2.5 *Data analysis*

The difference in cortisol secretion (ng ml<sup>-1</sup>) and cell yield (the number of wells plated) between the two methods was compared using a one-way analysis of variance (ANOVA) and followed by Student's t-test ( $\alpha=0.05$ ).

## **3.3 Results**

Cortisol secretion in response to ACTH stimulation in the new method was considerably higher than the original method, as shown in Table 3.1. The cell yield in the new method was slightly higher than the original, although this trend was not statistically significant. A considerable benefit of the new method is that cell aggregations do not form in the cell suspension, thus enabling the researcher to use less mechanical force during cell dispersion ensuring that receptor integrity is maintained. Additionally, the new method can be used with fish  $\geq 200$  g, whereas the original method was unable to produce a cell culture capable of measurable cortisol secretion in response to ACTH stimulation when fish of this size were used.

Table 3.1 Comparison between original<sup>a</sup> and modified head kidney primary cell culture methods

		Original Method	Modified Method
Technique	Tissue & solutions on ice	No	Yes
	Digestion temperature	23°C	21°C
	Digestion time	1 hr	2 hrs
	Centrifugation temperature	15°C	4°C
	Medium	MEM	HBSS, Ca & Mg free
	EDTA	No	Yes
Performance	Suitable for fish $\geq 200$ g	No	Yes
	Clumping	Yes	No
	Cell yield (number of wells)	$13.4 \pm 3.78^b$	$14.3 \pm 2.60$
	Avg. secretion (ng ml <sup>-1</sup> )	$18.6 \pm 5.10^*$	$31.1 \pm 11.4^*$
	Max. secretion (ng ml <sup>-1</sup> )	23.6	43.3

<sup>a</sup> (Leblond and Hontela, 1999), <sup>b</sup> cell yield and secretion data are presented as mean  $\pm$  standard deviation (n=5 for original method and n=8 for modified method),  $t(11)=2.28$ ,  $p=0.04$ . Asterisks denote that two means are statistically different ( $\alpha=0.05$ ).

### 3.4 Discussion

Two primary cell culture methods for the head kidney of rainbow trout were compared. The modified *in vitro* cell culture preparation was superior to the method developed by Leblond and Hontela (1999), because cell aggregations were reduced, the cells secreted more cortisol in response to the ACTH challenge test, and measurable secretion was achieved in fish  $\geq$  than 200g. Increased cortisol secretion in the new method was likely due to the use of a Ca<sup>2+</sup> and Mg<sup>2+</sup> free 0.02% EDTA solution and a longer digestion period.

A two hour digestion compared to a one hour digestion proved to be more effective for dissociating cells from the extracellular matrix, without affecting the integrity of ACTH surface receptors. Endocrine cells are very sensitive and special care must be taken not to damage the tissue when dissociating the cells. Enzymatic digestions are typically superior to mechanical methods, as they have proven to be less damaging to the cells (Waymouth, 1974). In conjunction



with enzymatic tissue dispersion, the use of a chelating agent such as EDTA and/or  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free media can further eliminate cell-to-cell adhesion.

The use of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free media reduces the likelihood that cell aggregations will occur in the cell suspension, as both of these ions play a role in cell-to-cell adhesion (Waymouth, 1974; Takeichi, 1990). EDTA is widely used as a chelating agent in cell culture preparations. It is involved in the sequestering of metal ions, helping to prevent the joining of cadherins (cell adhesion molecules), thereby maintaining cells in suspension (Waymouth 1974; Flora and Pachauri, 2010). A considerable amount of mechanical force can be necessary to disperse clumps of cells in suspension, which can damage surface receptors, leading to decreased cortisol secretion in response to ACTH stimulation. Additional changes in the new method included keeping tissue on ice, the use of ice cold solutions, a lower digestion temperature, and centrifugations at  $4^{\circ}\text{C}$ , likely helped to prevent cell damage and cell death, possibly contributing to the increase in cortisol secretion.

An increase in cortisol secretion is significant, because this means the cell density (number of cells  $\text{ml}^{-1}$ ) at which cells are typically plated, can be lowered. When conducting *in vitro* toxicity experiments on the effects of two or more chemicals, a large number of wells are required. If the cell density of the suspension is decreased, the number of wells yielded would potentially increase by 30-40%, making it more feasible to conduct mixture experiments. A limitation of the original method is the lower cortisol secretion and the difficulty to obtain an adequate number of wells to conduct mixture experiments at the cell density typically plated ( $75 \times 10^6$  cells  $\text{ml}^{-1}$ ).

### **3.5 Conclusion**

The protocol for the head kidney cell preparation developed by Leblond and Hontela (1999) was modified and its performance found to be superior, as determined by qualitative and quantitative analysis.

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**CHAPTER 4. THE EFFECTS OF TRICLOSAN, 2,4-D, AND THEIR BY-PRODUCTS ON  
THE ADRENOCORTICAL CELLS OF RAINBOW TROUT,  
*ONCORHYNCHUS MYKISS***

**4.1 Introduction**

A pressing issue in water quality research is the presence of endocrine disrupting compounds in surface water and their effect(s) on aquatic and human life. A number of different water-borne chemicals have endocrine disrupting effects including pharmaceuticals, pesticides, industrial chemicals, and heavy metals (Witorsch and Thomas, 2010; Corcoran et al., 2010; Shenoy and Crowley 2011). Endocrine disrupting compounds (EDCs) are defined by Crisp et al. (1998) as: “exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior” (p.17).

During the wastewater treatment process, personal care products, and pesticides are only partially removed and will be discharged with effluent into receiving waters (Kuster et al., 2008). In addition to water discharged from WWTPs, other sources of water-borne toxicants may include wastewaters from livestock operations, agriculture, and industry. These complex mixtures of chemicals are continually released into surface waters, where the fate and effects of many of these chemicals in the aquatic environment remain relatively unknown.

This study focused on four chemicals typically detected in surface water: the antimicrobial Triclosan (TCS), detected up to 2.3  $\mu\text{g l}^{-1}$  (Kolpin et al., 2002; Morrall et al., 2004), the herbicide 2,4 dichlorophenoxyacetic acid (2,4-D), detected up to 439  $\mu\text{g l}^{-1}$  (Anderson, 2005; Gibson et al., 2007; Kuster et al., 2008), and their by-products, methyl-Triclosan (M-TCS) and dichlorophenol (DCP), detected up to 10  $\text{ng ml}^{-1}$  and 19.96  $\mu\text{g l}^{-1}$ , respectively (Lindstrom et al., 2002; Andresen et al., 2007; Gao et al., 2008). The study focused specifically on their potential for endocrine disruption *in vitro*, using primary cultures of rainbow trout interrenal cells. The interrenal tissue (analogous to the mammalian adrenal gland) of teleost fish includes, among other

cell types, adrenocortical cells and plays an integral role in the regulation of growth, immune function, reproduction, and osmoregulation (Norris and Carr, 2006). Primary cultures of rainbow trout adrenocortical cells have been used to test for potential endocrine disrupting activity of various water-borne toxicants, because the hypothalamo-pituitary-interrenal axis is a vulnerable target (Hontela and Vijayan, 2009).

TCS is a broad-spectrum antibacterial and antifungal, with a number of commercial applications, although it is used primarily in personal care products such as hand soap, dish detergent, deodorant, toothpaste, plastics, and textiles (for review see Chapter 2). As consumers continue to demand products that offer ongoing antibacterial protection, manufacturers are increasing their use of TCS in common household products, despite the fact that several studies have called into question the efficacy of TCS in inhibiting the proliferation of bacteria (Kalyon and Olgbub, 2001; Junker and Hay, 2004; Aiello et al., 2007). The majority of products containing TCS are washed down household drains, eventually making their way to municipal WWTPs, where approximately 5% of antimicrobial is discharged into receiving waters (Singer et al., 2002). TCS has been measured in fish tissues, human plasma, breast milk, and urine, indicating a ubiquitous presence in surface water (Wolff et al., 2007; Calafat et al., 2008).

2,4-D, a broad-leaf phenoxy herbicide synthesized from chloroacetic acid and DCP, is one of the most widely used herbicides in the world. A synthetic auxin, 2,4-D disrupts protein synthesis and mitosis, causing abnormal growth patterns, and eventually tumours (Brook, 2008). In Alberta, 2,4-D accounted for almost 21.1% of pesticide sales in 2003, which translates into 763 357.6 kg of the herbicide (Byrtus, 2007). Agricultural applications are the primary source of 2,4-D release into the aquatic environment. Pesticide drift and runoff into groundwater and surface water is not uncommon. However, 2,4-D is a versatile pesticide and is used for a number of non-agricultural applications, e.g. forestry, turf, aquatic weeds (Bus and Hammond, 2007).

Once in the aquatic environment, both TCS and 2,4-D undergo degradation processes, producing several by-products. TCS undergoes photolysis and biological methylation to produce

DCP and M-TCS, respectively; 2,4-D produces DCP as one of its primary by-products. The detection rate of DCP in surface water is similar to those of its parent-compounds (House et al., 1997). Due to its  $K_{ow}$  value of 3.06, DCP is expected to adsorb to particulate matter and sediment. A study on the bioconcentration potential of DCP by Kondo et al. (2005) concluded that the chemical displays a low to moderate potential for bioconcentration. M-TCS is more environmentally persistent than TCS, probably the result of its greater lipophilicity (Chu and Metcalfe, 2007; Coogan et al., 2007). In general, water-borne toxicants with a high molecular weight and a  $\log K_{ow} > 5$ , such as M-TCS, have a tendency to partition into sediment, and are not commonly detected in surface water.

The effects of TCS and 2,4-D on aquatic organisms have yet to be clearly delineated. In fish, amphibians, and mammals, TCS can act both androgenically (Foran et al., 2000; Kumar et al., 2008; 2009; Zorilla et al., 2009) and estrogenically (Ishibashi et al., 2004; Matsumara et al., 2005; Raut and Angus, 2010). Additionally, several studies have demonstrated a potential for TCS to disrupt thyroid hormones (Veldhoen et al., 2006; Crofton et al., 2007; Paul et al., 2010). Similar to other pesticides, 2,4-D is suspected of disrupting thyroid homeostasis (Charles et al., 1996;

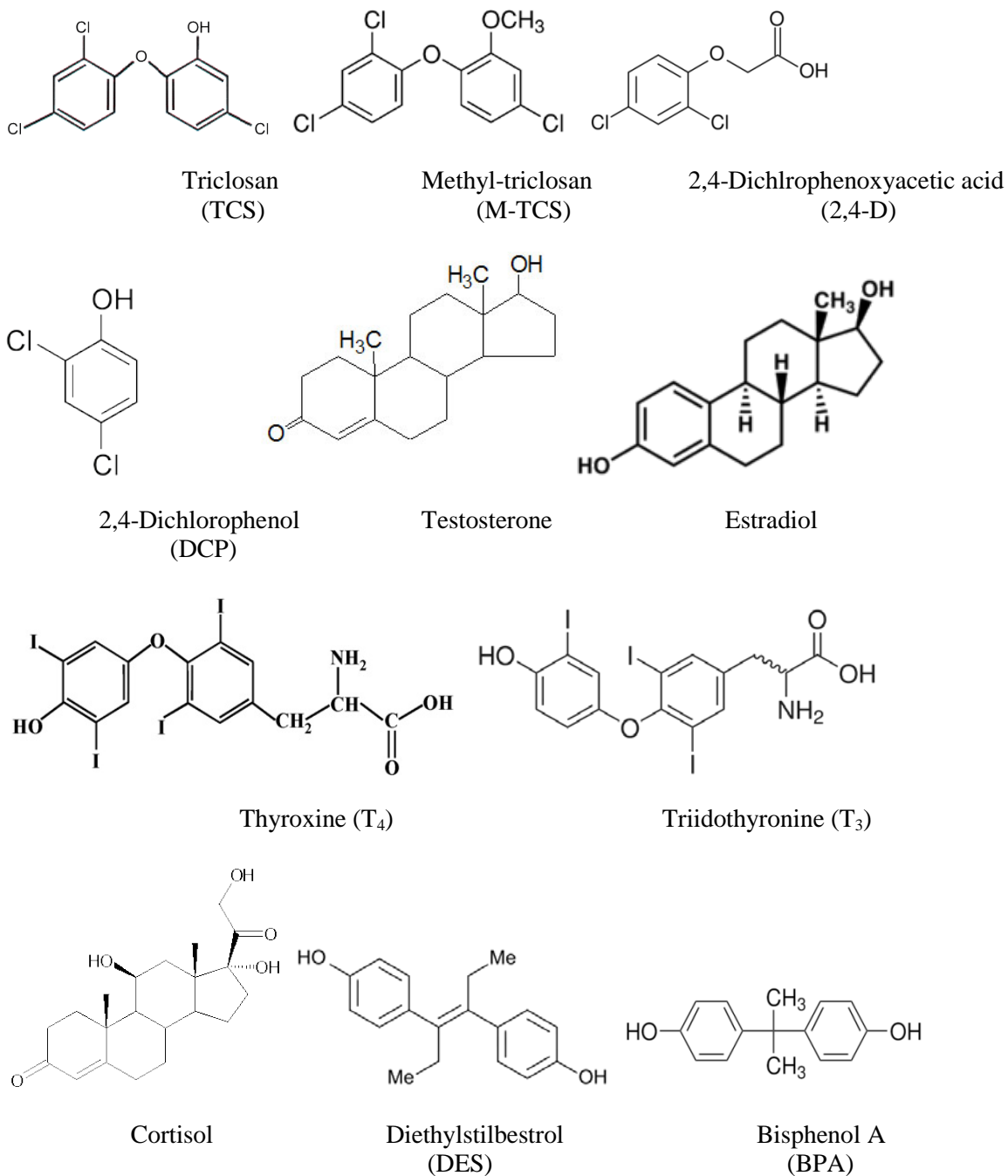


Figure 4.1. A structural comparison of TCS, M-TCS, 2,4-D, and DCP to endogenous hormones and known endocrine disruptors.

Rawlings et al., 1998; Kobal et al., 2000; Raldua and Babin, 2009). The herbicide impedes normal sperm development (Amer and Aly, 2001), induces testicular changes in rats (Oakes et al., 2002), increases androgen-dependent tissue weights (Kim et al., 2002), and exhibits androgenic

activity *in vitro* (Kim et al., 2005). Human evidence suggests that pesticide applicators exposed to 2,4-D have poor quality sperm, increased levels of lutenizing hormone (LH) and are more likely to have children with birth defects (Garry et al., 2001). As for the estrogenic potential of 2,4-D, it would appear that the reagent grade 2,4-D does not display any estrogen-like activity, while the commercial grade herbicides, 2,4-D LV4 and 2,4-D amine, do (Lin and Garry, 2000). These results indicate that the inert ingredient(s) in the pesticide formulations were responsible for the estrogenic endocrine disruption, highlighting the importance of testing commercial pesticide formulations.

In contrast to their parent-compounds, much less is known about the toxicity of the by-products DCP and M-TCS. Results from studies by Jobling et al. (1995) and Kramer and Giesy (1999), failed to uncover any estrogenic effects. Weak estrogenic activity was detected by Jones et al. (1998), Nishihara et al. (2000), and Han et al. (2002). There is a burgeoning body of evidence to suggest that the endocrine disrupting potential of DCP stems from the chemical's ability to act as an AR antagonist (Kim et al., 2002; 2005; Li et al., 2010). This makes DCP a potential threat to aquatic life, because the exposure of fish to anti-androgens is associated with the induction of intersex, decreased spermatogenesis, and lower sperm counts (Kiparissis et al., 2003). As for the by-product M-TCS, to date, there have been no studies investigating its toxicity. Aquatic organisms are consistently exposed to complex mixtures of water-borne chemicals, released from a number of anthropogenic source(s). The chemicals and their by-products investigated in this study are commonly detected in surface waters and the concentrations tested are those actually measured in the environment. They all contain phenolic moieties, and based on the findings that similar types of chemicals have endocrine disrupting effects, this study tested their potential to disrupt the synthesis of cortisol in a primary cell culture of rainbow trout adrenocortical cells.

## **4.2 Materials and Methods**

#### 4.2.1 Chemicals

Triclosan and M-TCS were obtained from Ciba Speciality Chemicals (Mississauga, Ontario). DCP was purchased from Sigma-Aldrich (Oakville, Ontario). 2,4-D ester was a generous gift from Dr. C. Sheedy at the Lethbridge Research Station. Multi-well (96-well) tissue culture plates were purchased from Fisher Scientific (Ottawa, Ontario). Tissue culture media, digestion enzymes, adrenocorticotrophic hormone (Porcine adrenocorticotropin, ACTH 1-39, Sigma), bovine serum albumin, sodium bicarbonate, and lactate dehydrogenase (LDH) assay kits were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario). Cortisol radioimmunoassay kits were purchased from MP Biomedicals (#07-221102, Medicorp, Montréal, Canada).

#### 4.2.2 Fish

Male and female juvenile rainbow trout (*Oncorhynchus mykiss*), body weight (162.1±14.0 g), were obtained from the Allison Creek Brood Trout Station (Crowsnest Pass, Alberta, Canada). Fish were maintained in a 1500-L flow-through tank at 13-15°C, on a photoperiod of 12L: 12D. Fish were fed to satiation with commercial trout chow food. Prior to the commencement of tissue harvesting, fish were acclimated in the laboratory for a period of two weeks.

#### 4.2.3 Preparation of adrenocortical cell suspensions

Animal use protocols were approved by the University of Lethbridge Animal Care Committee in accordance with national guidelines. Fish were anesthetized with MS-222 (0.5 g l<sup>-1</sup> of water) buffered with sodium bicarbonate to a pH of 7. To remove a maximum of blood from the head kidneys, fish were bled with a 1-cc heparinized syringe from the caudal vein, then perfused through the heart with 30 ml of a 0.7% saline solution, as described previously (Leblond and Hontela, 1999). The head kidney was excised and the tissue placed in ice cold medium, 0.02% Ethylenediaminetetraacetic acid disodium salt (EDTA) prepared in Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca<sup>2+</sup> and Mg<sup>2+</sup>. Prior to enzymatic digestion, tissue fragments were rinsed with ice cold 0.02% EDTA and minced into 1-mm<sup>3</sup> pieces. Tissue was digested in a



shaker incubator for two hours at 21<sup>0</sup>C. During digestion, medium was stirred with a transfer pipette every 10 min to facilitate tissue dispersion.

Digested tissue was washed with 0.02% EDTA and filtered through nylon mesh (30 μm pore size). Undigested tissue was mechanically dispersed with a plastic transfer pipette and the cell suspension filtered. The pellet was re-suspended in 1 mL of 0.02% EDTA and the cell yield and viability determined with trypan blue. Following plating, cells were centrifuged for 5 min at 200 g and re-suspended in MEM for a two hour incubation period prior to ACTH stimulation. The cell suspension was adjusted to a cell density of 75 X 10<sup>6</sup>, and plated at a volume of 100 μL per well in a 96-well microplate. Cells were pre-incubated for 1 hour.

#### 4.2.4 Toxicant Exposure

After the pre-incubation period, cells were centrifuged and MEM removed. Cells were exposed to the 100 μL of a single toxicant (TCS, 2,4-D, M-TCS or DCP) or a binary mixture of toxicants (TCS/2,4-D, DCP/2,4-D, or M-TCS/TCS) for one hour. Cells were centrifuged, and supernatant collected and stored at -81<sup>0</sup>C for an *in vitro* LDH based cell viability assay.

#### 4.2.5 Stimulation of Cortisol Secretion

After centrifugation, cells were washed in a balanced salt solution to remove any toxicant residue, and were re-suspended in 100 μL of MEM containing 1 U ml<sup>-1</sup> ACTH. Cells were incubated in an environmental chamber for 1 hour at 15<sup>0</sup>C. Following the one-hour stimulation, cells were centrifuged for 5 min at 200 g and the supernatant collected and frozen at -80<sup>0</sup>C. Cortisol concentration (ng ml<sup>-1</sup>) was measured in 75 μL of the supernatant with the radioimmunoassay, as described previously (Leblond and Hontela, 1999).

The LDH assay was used to measure cell viability. LDH release into the medium is an indicator of a loss of membrane integrity and cell death. Following toxicant exposure, supernatant was collected and stored at -80<sup>0</sup>C until analysis. Samples were pipetted at two volumes, 5 and 10 μL, into a 96-well microplate and the Lactate Dehydrogenase Mixture added to each well. Plates

were incubated in the dark, at room temperature, for 20 min. The reaction was terminated by the addition of 1N HCl. The absorbance was measured in a microplate reader at two wave-lengths, 490 and 690 nm. Cell viability was expressed as a percentage of the lysis treatment, with maximum LDH released in the medium used as 100% cell death.

#### 4.2.6 Data Analysis

Statistical analyses were performed using JMP Version 8 and data expressed as mean  $\pm$  standard error. All raw cortisol data ( $\text{ng ml}^{-1}$ ) from the cortisol radioimmunoassay was converted to a percentage of control (cells not exposed to the toxicant), with cells from one fish representing one replicate ( $n=1$ ). For the single toxicants, a one-way Analysis of variance (ANOVA) was performed, while a two-way ANOVA was used for the binary mixture experiments. Dunnett's test was used to compare the effect of single toxicant treatments to the control. Tukey's HSD was used to analyze the binary mixture data to determine how the addition of the second toxicant affected the toxicity of the first chemical. A probability level of  $p < 0.05$  was considered statistically significant.

### 4.3 RESULTS

#### 4.3.1 Single toxicant exposure

##### 4.3.1.1 Cortisol secretion

The endocrine disrupting potential of the toxicants TCS, M-TCS, 2,4-D, and DCP was determined *in vitro* using a rainbow trout adrenocortical cell bioassay. The cells were exposed to each toxicant at concentration of 0.01-110  $\mu\text{M}$  for 60 min. The functional integrity of the cells was assessed by the capacity to respond to ACTH and secrete cortisol, and cell viability was estimated by LDH release. The dose response curves for each single toxicant are shown in Figure 4.2. The half maximal effective concentrations ( $\text{EC}_{50}$ ) for the single toxicants are indicated in Figure 4.2. All four single toxicants significantly inhibited cortisol secretion in rainbow trout adrenocortical cells ( $p < 0.05$ ) (Figure 4.2.). The maximum inhibiting potencies of the individual chemicals are as follows; 67% (2,4-D), 65% (DCP), 42% (TCS), 32% (M-TCS), with  $\text{EC}_{50}$

ranked as follows: 2,4-D, DCP, TCS, M-TCS. Furthermore, at low, environmentally relevant concentrations of TCS (0.01 $\mu$ M) and 2,4-D (0.01-1 $\mu$ M), corticosteroid producing cells were unable to produce normal amounts of cortisol, following exposure to ACTH.

Comparisons of calculated EC<sub>50</sub> values for the parent-compounds, TCS and 2,4-D, and their by-products, indicate that the parent compounds are more potent inhibitors of cortisol secretion than their by-products, M-TCS and DCP, respectively. Despite differing toxicities, the parent compounds and their by-products exhibited similarly shaped dose-response curves, as illustrated in Figure 4.2., suggesting the parent compounds and their by-products display related chemical behaviour. However, it is important to acknowledge the possibility that the marked decrease in corticosteroid production at the maximum concentration of 2,4-D and DCP tested (100 $\mu$ M), could in part be due to the interference of these chemicals with the cortisol radioimmunoassay. Further testing is required to address this limitation.

#### 4.3.1.2 Cell Viability Assay

The cell lysing activity of the toxicants was generally quite low, rising slightly with increasing toxicant concentration (Figure 4.2.). For the single toxicant experiments, there was no difference in cell viability between the toxicant treatments and the control for MTCS and DCP ( $p < 0.05$ ). In the TCS experiment, the 10  $\mu$ M treatment was significantly different from the control, resulting in 8% cell death. The exposure of cells to 44  $\mu$ M of 2,4-D resulted in 6% cell death, a rate which was significantly different from the control, although far too low to explain the inhibition of cortisol secretion.

#### 4.3.2 Exposure to binary mixtures

##### 4.3.2.1 Cortisol secretion

The endocrine disrupting potential of binary mixtures of the parent compounds and their respective by-products is shown in Figure 4.3. Maximum inhibiting potencies of a binary mixture of the two parent-compounds, and each parent compound with their respective by-product ranged from 75% (TCS/MTCS), 56% (2,4-D/DCP), to 48% (2,4-D/TCS) (Figure 4.3.). In all three of the

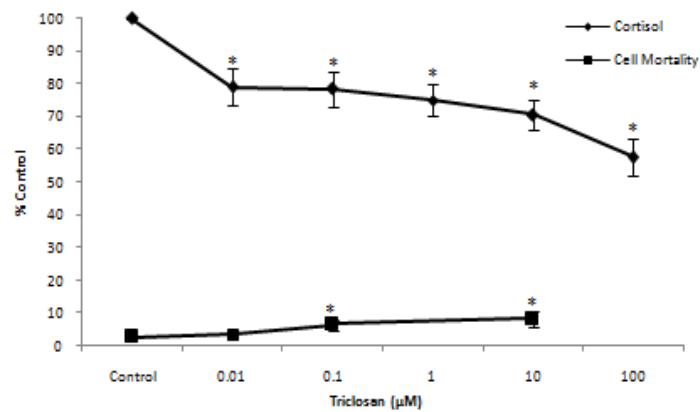
binary mixture experiments, the addition of the second toxicant failed to alter the inhibiting potency of the EC<sub>50</sub> of 2,4-D or TCS ( $p < 0.05$ ). Based on these results, the binary mixtures tested did not additively or synergistically decrease cortisol secretion. A limitation of the binary mixture experiment is that the concentration of 2,4-D and TCS tested may have been too high to detect a change in toxicity, with the addition of the second chemical. Future studies should test a wider range of toxicant concentrations to ensure, that if present, additive effects would be detected.

#### 4.3.2.1 Cell Viability Assay

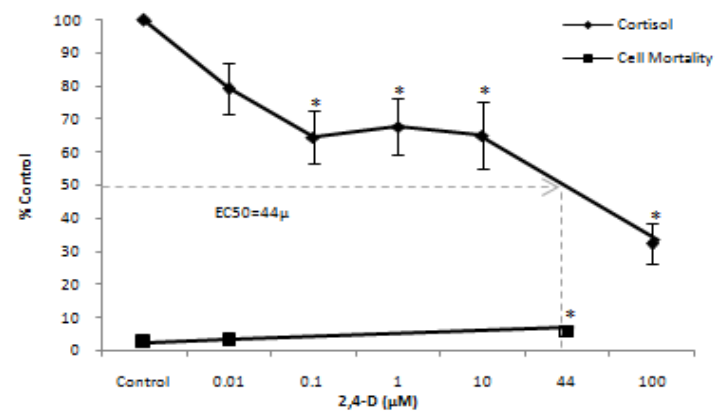
Cell viability in the binary mixture experiments did not decrease with the addition of a second toxicant ( $p < 0.05$ ), as shown in Figure 4.3. In both the 2,4-D/TCS and 2,4-D/DCP experiments, there was no difference in cell mortality between the control and toxicant exposed groups. In the TCS/MTCS experiment, only TCS alone (110  $\mu$ M) and TCS with 10  $\mu$ M M-TCS were significantly different from the control. Cell death in the binary mixture experiments did not exceed 16%, and cannot solely account for the observed decreases in cortisol secretion.

Figure 4.2. Cortisol secretion and cell mortality (% of control) of rainbow trout adrenocortical cells exposed to a) TCS;  $F(5, 110)=6.49$ ,  $p=0.0001$ , b) 2,4-D;  $F(6, 74)=8.28$ ,  $p=0.0001$ , c) M-TCS,  $F(4, 39)=4.52$ ,  $p=0.0048$  and d) DCP;  $F(4,38)=6.33$ ,  $p= 0006$ , *in vitro* for 60 min. Data is expressed as a % (mean $\pm$  standard error) of control (cells receiving no toxicant; \* indicates a significant difference,  $p < 0.05$ ).

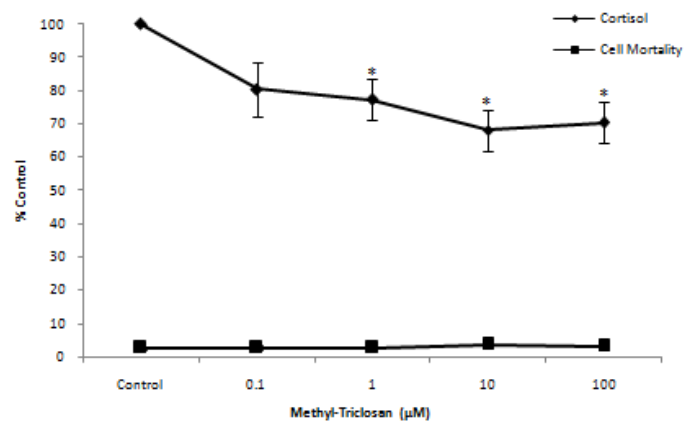
a) TCS



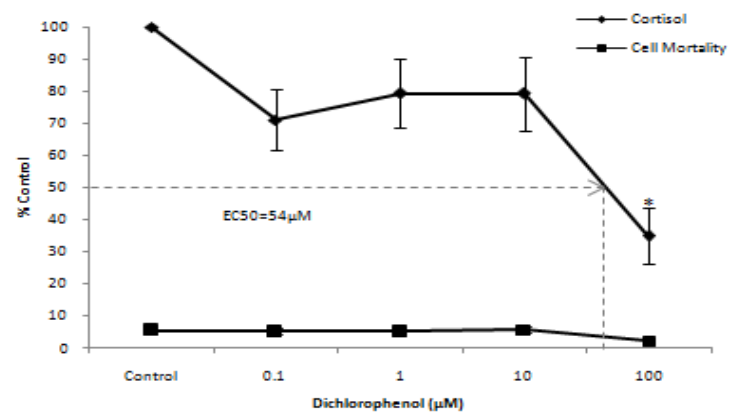
b) 2,4-D



c) M-TCS



d) DCP



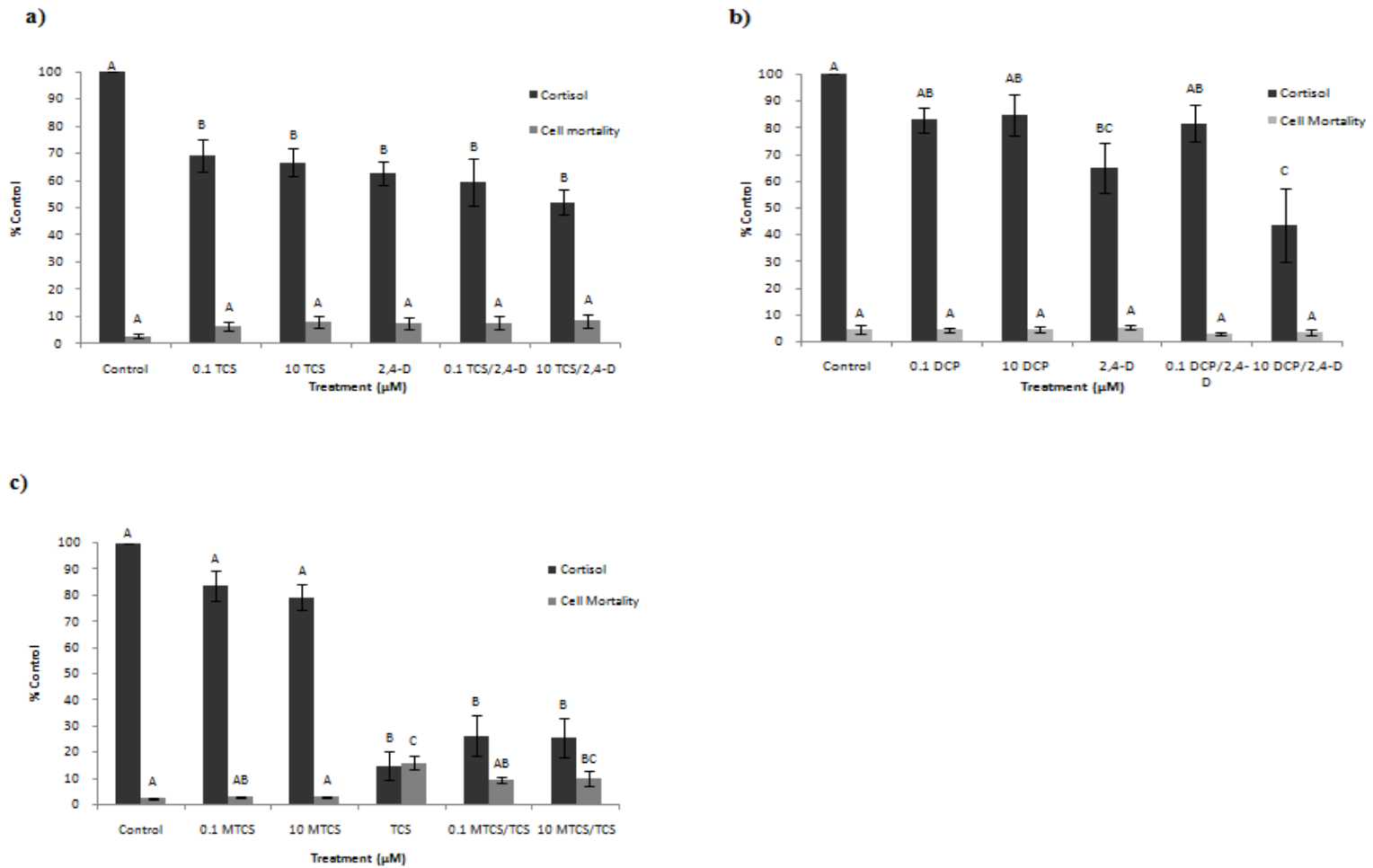


Figure 4.3. Cortisol secretion and cell mortality (% of control) of rainbow trout adrenocortical cells exposed to binary mixtures of toxicants a) TCS and 2,4-D ( $44\mu\text{M}$ );  $F(5, 47)=10.25$ ,  $p=0.0001$ , b) DCP and 2,4-D ( $44\mu\text{M}$ );  $F(5, 35)=6.79$ ,  $p=0.0002$ , c) MTCS and TCS ( $110\mu\text{M}$ );  $F(5, 65)=40.11$ ,  $p=0.0001$  *in vitro* for 60 min. Data is expressed as a % (mean  $\pm$  standard error) of control (cells receiving no toxicant); \* indicates a significant difference,  $p < 0.05$ .

#### 4.4 DISCUSSION

Endocrine disrupting compounds alter the production, release, distribution, and/or elimination of endogenous hormones. Pathways of endocrine disruption include interactions with membrane receptors, the aryl hydrocarbon receptor, enzymes involved in hormone synthesis, and the influence of these compounds on nuclear hormone receptors (le Maire et al., 2010). The objective of this study was to simultaneously assess the capacity of individual and binary mixtures of two commonly detected water-borne toxicants, TCS and 2,4-D, and their by-products, M-TCS and DCP, to disrupt corticosteroid production in a primary cell culture of rainbow trout adrenocortical cells. It was hypothesized, based on evidence from studies investigating chemically-induced disruption of hormonal systems (Colburn, 2004; Devillers et al., 2006; Kumar et al., 2008) that the exposure to these chemicals would inhibit cortisol secretion. In comparison to chemicals that alter estrogen and androgen synthesis and action, much less is known about non-sex steroid endocrine disruption. There is a substantial need to determine the extent to which water-borne toxicants are capable of disrupting the production of cortisol, because this hormone plays a pivotal role in the regulation of an animal's physiology (Mommsen et al., 1999). The cell model used in this study is an excellent screening tool for the assessment of the endocrine disrupting activity of chemicals typically detected in surface water, allowing for comparative assessments of single toxicants, binary mixtures, and complex mixtures. Once a potential for disruption of the corticosteroid pathways has been established, mechanistic studies can be conducted to elucidate the mechanism(s) of altered steroid hormone production.

A cell culture of fish adrenocortical cells was selected for this study even though the H295 cell line, a human adrenocortical carcinoma cell line, is a well-accepted *in vitro* model for assessing endocrine disruption to the HPA axis (Hecker and Geisy, 2008). This *in vitro* model has been used successfully in studies that profiled the cortisol disrupting effects of a number of different water-borne toxicants (Hontela and Vijayan, 2009; Miller and Hontela, 2011), including pesticides such as atrazine, mancozeb, diazinon, and endosulfan (Bisson and Hontela, 2002). In



the present study, all four chemicals alone and in binary mixtures, inhibited rainbow trout adrenocortical cells from synthesizing normal amounts of cortisol in response to the ACTH challenge test.

To determine whether the inhibition of cortisol secretion was primarily the result of endocrine disruption, it was necessary to rule out the possibility of cell death or cytotoxicity. In all four single toxicant exposures, cell death was never greater than 10%. In cells receiving the highest concentration of toxicant, cortisol secretion decreased by 30-60%, and could not be accounted for by cell death alone (see Figure 4.2.). The cytotoxicity of binary mixtures of TCS, 2,4-D, and their by-products exhibited similar results to the single toxicant experiments, with cell mortality remaining low, while cortisol secretion was inhibited by up to 85% (see Figure 4.3.). Based on the results from the cell viability assay, it was concluded that the decrease in the ability of these cells to respond to ACTH was due primarily to endocrine disruption, not cell death.

A limitation of many *in vitro* based endocrine disruption screening assays is that the concentration of toxicants typically tested is far greater than what is measured in environmental matrices. As a result, the environmental relevance of these studies often comes into question. In this study, environmentally relevant concentrations of TCS (0.01  $\mu\text{M}$  or 2.89  $\mu\text{g l}^{-1}$ ) and 2,4-D (0.1  $\mu\text{M}$  or 22.1  $\mu\text{g l}^{-1}$ ) were used and produced considerable disruption of cortisol secretion. These concentrations are within the range of those reported in surface waters, specifically 2.3  $\mu\text{g l}^{-1}$  for TCS (Kolpin et al., 2002; Morrall et al., 2004), and 439  $\mu\text{g l}^{-1}$  for 2,4-D (Anderson, 2005; Gibson et al., 2007; Kuster et al., 2008).

The chemicals screened for endocrine disruption in this study all contain a phenolic moiety, a characteristic which has become a hallmark for endocrine disruption (Devillers et al., 2006). It was hypothesized that based on the structural similarities of these chemicals to known endocrine disrupters, the chemicals tested would display some degree of hormone perturbation. Corticosteroid production is stimulated by ACTH, responsible for increases in cholesterol uptake into the steroidogenic cells, facilitating the movement of cholesterol into the mitochondria, and

activating the steroidogenic enzymes, including steroid acute regulatory protein (StAR) and the cytochrome P-450-mediated cholesterol side-chain cleavage enzyme (P450<sub>scc</sub>). Endocrine disrupting chemicals can influence any one, or more, of these key steps, thereby inhibiting the production of corticosteroids.

Based on what is currently known of endocrine disrupting compounds, alterations in corticosteroid production caused by TCS, M-TCS, 2,4-D, and DCP may occur as the result of a decrease in cholesterol production, decrease in the expression of StAR protein, or reduced expression of a number of key steroidogenic enzymes (Odermatt et al., 2006). Previous studies have shown that these toxicants, with the exception of M-TCS, affect thyroid hormones, the estrogen receptor, and/or the androgen receptor (Han et al., 2002; Raldua and Babin, 2009; Li et al., 2010; see review by Dann and Hontela, 2011). Although many of the studies on endocrine disruption have focused on StAR protein (Murugesan et al., 2007; Kitamura et al., 2005) and P450<sub>scc</sub>, other enzymes such as 17 $\alpha$ -hydroxylase, 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD), and 21- and 11 $\beta$ -hydroxylases (CYP11 $\beta$ ) (Miller, 1988; Lyssimachou and Arukwe, 2007) must not be overlooked. The synthesis of StAR protein and P450<sub>scc</sub> is governed by the acute release of tropic hormones such as ACTH or luteinizing hormone (LH), and occurs rapidly in all steroidogenic tissue (Arukwe, 2008).

In steroid producing cells, StAR protein funnels cholesterol from the outer mitochondrial membrane to the inner membrane for conversion to pregnenolone. The movement of cholesterol across mitochondrial membranes by StAR protein is widely considered to be the rate limiting step of steroidogenesis (Stocco, 2001). Two extensively used pesticides, lindane and dimethoate, block the expression of StAR protein, resulting in decreased levels of serum testosterone (Walsh et al., 2000; Walsh and Stocco, 2000). Other chemicals that specifically target StAR protein include: econazole (Walsh et al., 2000), glyphosate (Walsh et al., 2000), carbachol (Janossy et al., 2001), ethanol (Khisti et al., 2003), arsenite (Zhao et al., 2005), anisomycin (Zhao et al., 2005), bromocriptine (Kan et al., 2003), and spironolactone (Hischerova et al., 2004). More recently, a

study by Kumar et al. (2009) reported that TCS decreased transcription and translation of StAR and P450<sub>scc</sub> proteins in rat testes.

This finding is significant, as these proteins play a critical role in the steroidogenic cascade, and appear to be more sensitive to the toxic insults of endocrine disrupting compounds than enzymes further downstream from P450<sub>scc</sub> (Walsh et al., 2000). Geslin and Auperin (2004) have provided a clear link between cortisol genesis and the upregulation of StAR protein and P450<sub>scc</sub> in the head kidney of rainbow trout. Furthermore, in juvenile salmon exposed to pp-DDE and EE2, changes in the expression of StAR protein and P450<sub>scc</sub> in interrenal tissue were observed.

It is clear that the inhibition of several key enzymes involved in steroid hormone production can account for a substantial degree of hormone disruption observed both in vitro and in vivo. In addition to the inhibition of StAR protein and cytochrome P450<sub>scc</sub> enzyme, inhibition of 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) (Blaha et al., 2006), and 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) (Akingbemi et al., 2001; Xu et al., 2006) by endocrine disruptors have been well documented. Although the mechanisms of corticosteroid disruption by TCS, 2,4D and their by-products were not within the scope of the current study, future work should focus on the impacts of these chemicals on the expression and activity of key steroidogenic enzymes.

In conclusion, the commonly detected water-borne toxicants, TCS, 2,4-D, and their by-products, M-TCS and DCP, act, at environmentally relevant concentrations, as endocrine disruptors in primary cell cultures of rainbow trout interrenal cells. The endocrine disrupting potency, expressed as EC<sub>50</sub> for inhibition of cortisol secretion, of the tested chemicals was ranked as 2,4-D > DCP > TCS > M-TCS, indicating that the parent compounds are more potent than the by-products. There was no evidence for interactions between TCS and 2,4D, a mixture often detected in the aquatic environments. Endocrine toxicology relating to the adrenal gland is of paramount importance, because this gland is the most common target for endocrine disrupting compounds in vivo, yet has not received its fair share of attention in the literature. Future studies

are needed to determine at which level(s) of the hypothalamo-pituitary-interrenal axis these endocrine disrupting compounds are acting on, and the molecular and cellular mechanisms of their toxicity.

### **Acknowledgements**

This study was funded by NSERC Discovery Grant to A. Hontela and the Keith and Hope Memorial, Entrance and Continuing M.A./M.Sc., and Queen Elizabeth II scholarships to A. Dann. We thank L. Miller for help with the cell culture and R. Royer for help with fish husbandry.

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## CHAPTER 5. GENERAL CONCLUSIONS

Corticosteroids play a pivotal role in many physiological processes, including immunity, cognitive function, growth, metabolism, reproduction, mineral balance, and blood pressure. The ubiquitous presence of anthropogenic chemicals in surface water is a widespread problem, as many of these chemicals are capable of altering the normal biological functioning of endogenous hormones. Endocrine disrupting compounds can arise from numerous sources and may fall under the following classes of chemicals: pesticides, industrial chemicals, pharmaceuticals, personal care products, and/or heavy metals. The endocrine disrupting activity of two commonly detected water-borne toxicants, a personal care product, triclosan (TCS), a pesticide, dichlorophenoxyacetic acid (2,4-D), and their by-products, methyl-triclosan (M-TCS) and dichlorophenol (DCP), on the adrenocortical cells of rainbow trout was investigated. Although it has been shown in earlier studies that TCS, 2,4-D, and DCP exhibit a potential for endocrine disruption, it was unknown if these chemicals are capable of affecting corticosteroid balance. In this study, all four chemicals showed significant inhibitory effects on corticosteroid genesis, although there were considerable differences in their activity. The chemical that exhibited the highest endocrine-disrupting potency was 2,4-D, followed by triclosan (TCS), dichlorophenol (DCP), and methyl-triclosan (M-TCS). Both parent-compounds proved to be more toxic than their degradation products. More research with suitable test systems is needed to determine the mechanism(s) of action of these corticosteroid disruptors and the health risk that they may present.