Prelude & Objectives
PRELUDE

The release of organic contaminants of emerging concern such as endocrine disrupting chemicals, and pharmaceuticals and personal care products (PPCPs) into the environment has received increasing public concern recently. PPCPs include a large number of chemicals that can originate from human usage and excretion, veterinary applications of a variety of products, such as prescription/non-prescription medications, and fungicides and disinfectants used for industrial, domestic, agricultural and livestock practices. PPCPs and their metabolites are continually introduced into the environment and are prevalent at detectable concentrations (Kolpin et al., 2002), which may affect water quality and potentially impact drinking water supplies, and ecosystem and human health. There is a growing concern regarding the presence of pharmaceuticals and personal care products (PPCPs) in the environment and their possible subtle effects on non-target organisms (Daughton and Ternes, 1999).

Pharmaceuticals and personal care products (PPCPs) are a class of emerging environmental pollutants that, upon introduction into aquatic ecosystems, may act on molecules, cells and organs in different organisms through unexpected modes of action (Fent et al., 2006). Municipal wastewater is one of the major sources of environmental pollution for numerous organic contaminants such as endocrine disrupting chemicals and PPCPs. One group of PPCP that has received increasing attention is antimicrobials because of their pronounced microbial and algal toxicity and potential for fostering resistance (Levy et al., 1999; Schweizer, 2001; Wilson et al., 2003). Triclocarban (3,4,4′-trichlorocarbanilide or N-(4-chlorophenyl)-N-(3,4-dichlorophenyl) urea), one of the most used PPCPs worldwide whose increasing environmental levels are causing growing concern about their presence in the ecosystems (Binelli et al., 2009).

TCC, a polychlorinated phenyl urea pesticide, marketed under the trademark TCC are extensively used since 1957 in a wide variety of personal care products including antibacterial soaps antibacterial mouthwashes and detergents, toothpaste, shampoos, deodorants, cosmetics and skin care lotions as well as other consumers goods (Singer et al., 2002; Tsai et al., 2008). In a limited retail survey, approximately 45% of liquid and bar soaps on the market contained this antimicrobials; TCC were the predominant antimicrobial in 76% of liquid
soaps and 29% of bar soaps, respectively (Perencevich et al., 2001). These products normally contain 0.1–2% of TCC by weight in their formula (Liu, 2004; Chalew, et al., 2009). In the United States TCC has been added to such products at levels of 0.5–5.0 wt % for prevention of spoilage and microbial infection control (TCC Consortium, 2002a). Approximately 80% of antimicrobial bar soaps on the US market contain TCC (Perencevich et al., 2001). In 1998, TCC production for the US market was estimated to approach one million pounds or about 454 metric tons per year (TCC Consortium, 2002a).

TCC is structurally related to carbanilide compounds, including some drugs and pesticides, and sterically and electronically related to a variety of other chemicals. TCC is monitored under the USEPA High Production Volume (HPV) chemical (i.e. 227–454 Mg produced/imported year$^{-1}$) program, but a recent report identifies TCC as a Moderate Production Volume (MPV) chemical (i.e. <227 Mg produced/imported year$^{-1}$) (USEPA, 2009a). Physical and chemical properties of TCC estimated using the US EPA’s QSAR Software (EPI Suite) shows that they have low vapour pressure and low water solubility, suggesting that it is not volatile and not very water soluble compound. TCC solubility data are limited to early measurements (0.11 mg L$^{-1}$; Roman et al., 1957), inadequately described methods (0.11 or 11 mg L$^{-1}$; TCC Consortium, 2002b), or are estimated using QSAR analyses (0.65–1.55 mg L$^{-1}$). High Kow (octanol and water partition coefficient) and Koc (soil adsorption coefficient) values also suggest that they have a tendency to partition onto soil or sediment in the environment. Commonly reported log Kow values range from 4.2 to 6.0 (TCC Consortium, 2002a; Halden and Paull, 2005; Sapkota et al., 2007; Ying et al., 2007). Because of its high hydrophobicity, this compound would be expected to adsorb to particulate material (Orvas et al., 2002; Halden and Paull, 2005).

TCC is a subject of recent criticism by consumer advocate groups and academic researchers alike. Concerns primarily center on uncertainties regarding TCC environmental concentrations and fate (Heidler et al., 2006), persistence (Ying et al., 2007; Miller et al., 2008), toxicity (Heidler et al., 2006), bioaccumulation potential (Daughton and Ternes, 1999; Darbre, 2006; Coogan et al., 2007), endocrine effects (Chen et al., 2008), potential for antibacterial resistance development (Suller and Russell, 1999; Walsh et al., 2003) and bioconcentration, metabolism and excretion (Schebb et al., 2011). Studies examining the degradation potential of TCC are limited in
number, but all indicate TCC will likely persist in the environment. These reports include a one-soil laboratory microcosm experiment, for which the aerobic TCC half life was estimated to be 108 days, whereas under anaerobic conditions little TCC biodegradation was found within 70 days (Ying et al., 2007). Halden and Paull, (2005) reported that TCC is expected to persist in the environment, with a half-life of 120 days in soil and 540 days in sediment.

In addition, a recent study documented persistence of TCC in estuarine sediment cores taken near wastewater treatment plants (New York), with peak levels being on the order of 24,000 ng g⁻¹ (Miller et al., 2008). There have been few reports on the concentrations of TCC in sediments (Sapkota et al., 2007; Miller et al., 2008; Wilson et al., 2008). However, higher concentrations of TCC (700–1600 ng g⁻¹) were found in the Back River of the Chesapeake Bay watershed close to a sewage effluent discharge point (Miller et al., 2008). Although not specifically investigated several studies suggest that TCC will likely persist in agricultural soils following land application of biosolids. TCC are typically found at μg or mg kg⁻¹ (3,050 – 51,000 μg kg⁻¹) in biosolids (Kinney et al., 2006; Chu and Metcalfe, 2007; USEPA, 2009). A complete understanding of the concentration and fate of TCC in agricultural soils following land application of biosolids is needed because of concerns over bioaccumulation and movement to surface waters.

Despite extensive production of TCC over the past 49 yrs, its fate in wastewater and the environment has received only limited attention. Following typical use in personal care products, TCC is washed down the drain and commonly becomes a constituent of domestic wastewater at documented concentrations of 0.4–50 μg L⁻¹ (TCC Consortium, 2002a; Halden and Paull, 2004; Heidler et al., 2006). TCC have been detected in surface runoff and in tile drainage from land receiving both liquid and dewatered municipal biosolids (Topp et al., 2008; Edwards et al., 2009). This widespread use, reported at 0.6–10 million Kg yr⁻¹ (TSCA, 2003; Miller et al., 2008), is a cause for concern because of recent reports of incomplete TCC removal during wastewater treatment (Heidler et al., 2006; Chu and Metcalfe, 2007) and the detection of these chemicals in surface waters (Lindstrom et al., 2002; Halden and Paull, 2004, 2005; Sapkota et al., 2007; Young et al., 2008).
TCC may reach the environment due to their incomplete removal in wastewater treatment plants or direct discharge of wastewater without treatment (Chalew et al., 2007). Owing to their hydrophobic nature, TCC were found at 0.09–51 mg Kg$^{-1}$ levels in sludges (Heidler et al., 2006; Ying et al., 2007). The occurrence of TCC in wastewater treatment plant (WWTP) influent, effluent and surface waters has been well documented (Halden and Paull, 2004, 2005; Heidler et al., 2006; Coogan et al., 2007; Young et al., 2008). TCC have been reported in wastewaters and surface waters ranging from 9 ng L$^{-1}$ to 6.7 µg L$^{-1}$ (Kolpin et al., 2002; Halden and paull, 2005; Ying and Kookana, 2007, Zhang et al., 2007; Zhao et al., 2009).

Environmental concentrations of TCC have been reported as high as 5600 ng L$^{-1}$ in river water (Halden and Paull, 2005). TCC has been found in the 0.25 µg L$^{-1}$ level in surface waters and up to 7µg L$^{-1}$ in USA water resources and STPs (Chen et al., 2008; Higgins et al., 2009). TCC is a previously unrecognized environmental pollutant, likely present at detectable concentrations in about 60% of US streams nationwide and measured concentrations of up to 6750 ng L$^{-1}$ in Maryland streams and estimated concentrations of up to 1550 ng L$^{-1}$ for streams surveyed previously by the USGS (Halden and Paull, 2005). TCC was detected in most of samples from the Liuxi River, Zhujiang River and Shijing River (China) as well as the effluents from the four WWTPs recording highest concentrations of 338 ng L$^{-1}$ TCC in surface water and 2633 ng g$^{-1}$ in the sediment which were both found in Shijing River (Zhao et al., 2010). The concentration ranges of TCC (23.9–342 ng L$^{-1}$) in the effluents measured were similar to those reported in U.S., Australia, Japan and Switzerland (Singer et al., 2002; Halden and Paull, 2005; Heidler et al., 2006; Ying and Kookana, 2007; Nishi et al., 2008; Ying et al., 2009), but higher than those in Spain (Pedrouzo et al., 2009). The reported TCC concentrations ranged to 5600 ng L$^{-1}$ in Greater Baltimore area, U.S. (Halden and Paull, 2004, 2005).

TCC toxicity has been reported for a variety of mammals. Reproduction and offspring survival rates decrease in rats and rabbits in response to elevated TCC levels. Additionally, TCC is known to cause methemoglobinemia (“Blue Baby” Syndrome) in humans (Ponte et al., 1974; Nolen and Dierckman, 1979). Mono- and di-chlorinated anilines, environmentally persistent TCC breakdown products, are also known to express ecotoxicity, genotoxicity, and hematotoxicity (Gledhill, 1975; Boehncke et al., 2003).
Prelude and Objectives

The enhancement characteristics of TCC and its carbanilide analogs on endogenous/exogenous androgens and estrogens, in contrast to antagonist activity of TCS, may have an effect on possible normal physiology and/or reproduction in both males and females (Swan et al., 2007). Exposure to TCC together with the steroid hormones in women may result in similar poor sperm quality because testicular development may be altered in utero. An animal study has indicated that chronic oral administration of high doses of TCC to male rats resulted in testicular degeneration (Scientific Committee on Consumer Products, 2005).

The Hershberger assay for in vivo screening to evaluate the synergistic effect of testosterone by TCC demonstrated that the treatment of 0.25% (wt/wt) TCC mixed in rat chow in the presence of testosterone propionate (0.2 mg/kg by subcutaneous injection) significantly increased the weight of accessory sex organs and tissues of reproductive tracts of castrated male Sprague Dawley rats, compared with rats given testosterone treatment alone (Chen et al., 2008). Human exposure to TCC contained in commercial personal care soaps that are frequently used may enhance the activity of endogenous sex steroid hormones, suggesting that TCC as an EDC may affect male reproductive systems.

In females, because the breast can be exposed to antimicrobial TCC-containing products such as soap and deodorants applied to the underarm and breast area, TCC amplification of E2-induced ER activity may harm patients with ER-positive breast cancer. Similarly, < 1 μM, TCC showed no significant effect on methylthiazol tetrazolium activity in the HEK 2933Y-ARE cell proliferation assay (Chen et al. 2008).

The physicochemical properties of TCC suggest that it will penetrate the skin poorly, and this prediction is supported by limited experimental data (Halden and Paull 2005). However, soaps provide good emollients to accelerate dermal penetration of TCC. TCC being an antibacterial agent with broad spectrum activity is effective against bacteria commonly found on the skin, including pathogenic Staphylococcus species which are resistant to many bacteriostats. It has been shown to reduce body odour by preventing growth of the causative organisms. It is non-irritant and has a high affinity for the skin which means that the bacteriostatic effect persists after application. The low mammalian toxicity of TCC together with its antibacterial activity have given rise to its usage as a bacteriostatic agent in bar soaps for over 35 years and more recently in liquid soap.
products. TCC is described in the promotional materials of its commercial suppliers as having activity at low concentrations in finished products, typically in the range 0.5 to 1.5% w/w (Charlton and McGillycuddy, 2001). TCC has a very low water solubility (<0.1%) but are fat-soluble and easily cross cell membranes (Halden and Paul, 2005). This confers the advantage that once applied to the skin, it is not readily removed by rinsing.

So far there have still been few studies on the fate of this compound in the receiving aquatic environment and potential ecotoxicological risks. TCC a High-Production-Volume (HPV) chemical ranked to have high risks to aqueous organisms (USEPA, 2009). Such release of TCC has the potential to cause a number of environmental and human health problems, including: the bioaccumulation of TCC in algae and snails (Coogan et al., 2007; Coogan and La Point, 2008); algal growth inhibiting effects (Yang et al., 2008); the potential of TCC to act as an endocrine disrupting compound (Ahn et al., 2008; Chen et al., 2008); the formation of toxic degradation products (Gledhill, 1975; Chhabra et al., 1991; Aranami and Readman, 2007); and the development of microbial resistance (Heath et al., 1999, 1998, 2000; Hoang and Schweizer, 1999) suggesting potential risks to aquatic organisms at environmental concentrations. There is little published information on the aquatic toxicity of TCC.

Computer modelling using the PBT Profiler estimated fish bioconcentration factor values (log BCF 3.074 for TCC), suggesting that TCC has the potential to bioaccumulate. TCC can be toxic to aquatic organisms such as fish with chronic toxicity value of 0.9 µg L\(^{-1}\) for TCC as estimated by the PBT Profiler. Toxicological studies performed with TCC in fish indicate that acute and chronic toxicities are observed at concentrations of 49–180 and 5 µg L\(^{-1}\), respectively, noting that the chronic effect threshold is within the environmental concentrations reported for surface waters (Chalew and Halden, 2009). Interestingly, recent studies have classified TCC as a new type of endocrine disruptor that works synergistically to amplify the expression of testosterone, suggesting that TCC should be classified as a steroid hormone enhancer (Chen et al., 2008). These previous studies emphasize the importance of evaluating the relative benefits and risks of TCC found in personal care products. Therefore, to protect aquatic ecosystems, as well as drinking water supplies, there is a clear need to examine the toxicity of TCC.
In order to test this hypothesis and investigate its effect the present study was designed to use certain biomarkers based on their capability to respond to contaminant exposure, either specific or general responses. The biomarkers in liver chosen were: biotransformation enzymes *viz.*., ethoxyresorufin-O-deethylase (EROD) activity as phase I biotransformation parameter and glutathione-S-transferase (GST) activity as a phase II conjugation enzyme; morphological and physiological parameters *viz.*., hepatosomatic index (HSI) and hepatic histology; and gene expression of major nuclear receptors *viz.*., androgen receptor (AR), estrogen receptor (ERβ), glucocorticoid receptor (GR), thyroid receptor (TRα); chaperone protein gene *viz.*., heat shock proteins (HSP90 and HSP70); and vitellogenin gene (VTG).

To the best of our knowledge the above said parameters have not been throughout taken up or investigated so far to TCC effect in aquatic species.
OBJECTIVES

This study (sublethal chronic study, 42 days) was aimed to evaluate the potential effects of environmentally relevant concentrations TCC in the rare minnow, *Gobiocypris rarus* (♂ and ♀) with following objectives.

† To determine the median lethal concentration (LC50) of TCC for 96 h to rare minnow, *G. rarus*.

† To investigate the effects of TCC on EROD and GST activities in liver of rare minnow, *G. rarus* as they play a central role in the inactivation/detoxification of exogenous and endogenous substrates and has been used as potential biomarkers for exposure to different environmental pollutants.

† To assess gross morphometric index *viz.*, HSI and hepatic histology of mature male and female fish after exposure to sublethal concentrations of TCC as these parameters can offer ‘earlier’ warning signals in toxicological studies.

† To examine gene expression of *G. rarus* (♂ and ♀) steroid hormone receptors *viz.*, AR, ERβ and GR and to analyze the mechanisms of interactions between these receptors and the toxicant TCC.

† To investigate the mechanisms of the HPT axis responses to TCC *via* TRα and discuss the possible molecular mechanisms underlying toxic response particularly, after exposure to environmental relevant TCC concentration.

† To evaluate the relative expression of liver HSP70 and HSP90 of adult rare minnow and explore their roles in response to stress and chaperon role of it to steroid hormones receptors.

† To determine crosstalk between liver-specific VTG mRNA transcript levels, a highly sensitive biomarker and TCC, thereby to provide an insight about TCC being an endocrine disruptor.