CHAPTER I

BACKGROUND

Pharmaceutical drugs are produced and prescribed to cure the diseases, and to improve human health, agriculture and aquaculture (Dietrich et al., 2002; Zhang et al., 2008; Cunningham et al., 2009). Halling-Sørenson et al. (1998) reported that their production and use exceeds several hundred metric tons annually. A total of 170 pharmaceutical chemicals are estimated to be used in excess of 1 ton per year (Webb, 2000). A major inlet of these compounds in the environment is through excretion in both urine and feces (Kummerer et al., 2000; Farre et al., 2001; Heberer, 2002; Fent et al., 2006). These drugs enter into aquatic environment through domestic waste waters, disposal from medical centres, and excretion via water and sewage treatment systems (Sanderson et al., 2004; Rosal et al., 2008; Kar and Roy, 2010). Kummerer (2004a) reported that several pharmaceuticals widely used in human medicine is excreted unchanged or as active metabolites in high percentages, and continuously discharged in to domestic waste waters. Moreover, during sewage treatment, most of these compounds are not quantitatively removed and remain in the effluents that get into the surface and groundwater (Ternes, 1998; Doll and Frimmel, 2003).

The occurrence of pharmaceuticals in the environment is a growing concern (Daughton and Ternes, 1999; Kummerer et al., 2000; Heberer, 2002, Ramaswamy et al., 2011). The presence of pharmaceutical drugs in waterways has been concern of several publications (Daughton and Ternes, 1999; Ramaswamy et al., 2011), but increased attention is also currently focused on drug residues as potential pollutants. Among the various classes of pharmaceuticals, anti-inflammatory, antibiotics, antiphlogistics, antiepileptics, beta-blockers, lipid regulators, vasodilators, and sympathomimetics, have been detected in drinking water, groundwater, wastewater, sewage, manure at ng - µg/L (Halling-Sørensen et al., 1998; Kummerer et al., 2000; Ternes et al., 2002; Zwiener and Frimmel, 2004; Gros et al., 2006; Benotti et al., 2009; Kar and Roy, 2010; Ramaswamy et al., 2011).
The growing use of pharmaceutical products is becoming a new environmental problem. Environmental pollution by pharmaceuticals is a complex issue, involving thousands of different active molecules, belonging to various therapeutic classes, with different physico-chemical properties, chemical structures, environmental behaviour and persistence (Zuccato et al., 2006). They are biologically active compounds that may interfere with specific biological systems or generically act depending on their properties. Water-soluble compounds may contaminate waters because of their mobility, while lipophilic compounds may accumulate in sediments or soils. The ecotoxicological effects of pharmaceutical drugs on different levels of the biological hierarchy from bacteria to the entire biosphere are not well known.

Toxicological hazards measured by bioassay procedures are more realistic than those predicted from the results of chemical analyses and the available information on the toxicity of chemical compounds detected (Genjutulin, 1990). Bioassay tests can be used to establish the maximum acceptable concentration of a pollutant in a given environment without deliberate application of the chemical causing any unfavorable biological consequences (Cairns, 1984) and are accepted as standard methods for assessing toxicity of chemicals (APHA et al., 1985). Even slight, non-significant influences on single components within regulatory cascades, which would not result in any acutely discernible effect, might ultimately affect a whole population by their negative consequences on fitness: disturbances in hormonal homeostasis (endocrine disruption), immunological status, signal transduction or gene activation (Seiler, 2002; Jos et al., 2003).

In aquatic ecosystems, organisms are constantly exposed to different levels of physical and chemical stressors which may likely to reduce the fitness of organisms and could involve ‘ecological death’ (Scott and Sloman, 2004). To estimate and predict the effects, the need of relevant tools has considerably increased in the past few years simultaneously to contamination sources and types. In this general context of quality biomonitoring, selecting the most sensitive species/gender/life stage is a necessity since it allows a more suitable risk assessment of stress (Sornom et al., 2010). Fish have been widely used as bioindicators and biomonitors in various aquatic systems.
Primarily the aim of using bioassay tests in monitoring of environmental pollution is to establish a relationship between toxicity and concentration of a pollutant being studied in the biotope; the toxic effects can be divided into two categories viz., effects that occur very quickly after a brief exposure to a chemical agent (acute) and those that appear only after repetitive exposure to the substances (chronic) (Ramade, 1987; Nagel, 1993). In hazard and risk assessment, the ratio between acute and chronic toxicity is often taken as a parameter for risk evaluation.

Acute and chronic toxicity tests are widely used to evaluate the toxicity of chemicals on non-target organisms (Santos et al., 2010). Importantly, McLeese et al. (1982) stated that among toxicity tests, static and continuous flow system are widely used; static tests are useful for short-term exposure and serves as first check to evaluate acute toxicity of a chemical to a number of organisms. Especially in ecotoxicological studies, static bioassay tests have been widely used for evaluating the impacts of toxic chemicals on aquatic organisms (Doudoroff et al., 1951). Ferguson et al. (1966) reported that static toxicity test may be more realistic than continuous flow test in simulating natural conditions.

The decision whether a certain xenobiotic is dangerous for the aquatic system and the food cycle, can only be made after the (a) mammalian acute toxicity (b) bacteria acute toxicity (c) fish acute toxicity and (d) biological dissociation tests have been carried out in detail (Ardalı, 1990). For the biomonitoring of aquatic pollution, the organisms in the aquatic systems are sampled for the analysis of various biological responses to chemical exposures. Suitable bioindicators usually give great help to the biomonitoring (Zhou et al., 2008). Aquatic organisms have often been used in bioassays to monitor water quality (Carins et al., 1975). Aquatic organisms are particularly important targets, as they are exposed via wastewater residues over their whole life. Among aquatic species, fish are the inhabitants that cannot escape from the detrimental effects of pollutants (Olaifa et al., 2004). Therefore, much more should be known about potentially acute and chronic bioassay tests and effects of pharmaceuticals in the aquatic system.

Drugs, which have specific mode of actions, may probably exert effects on terrestrial and aquatic ecosystems, when released into the environment.
Importantly, fish is most susceptible to absorb small molecules dissolved in their habitants via chorion, skin and gills, and also administration of pharmaceutical drugs (Parng, 2005). Because fish can biomagnify contaminants, fish are potentially useful sentinels of aquatic environmental degradation. Powers (1989) suggested that fish models are increasingly used in the early phases of pharmaceutical development and its toxicity evaluation.

Among the various pharmaceutical drugs detected in the aquatic environment, clofibric acid (CA) \((\alpha-\text{(p-Chlorophenoxy)} \text{isobutyric acid}) \) and Diclofenac (DCF) \(2-[(2, 6\text{-Dichlorophenyl}) \text{amino}] \text{benzene acetic acid} \) sodium salt has been detected extensively in different water bodies. CA is an active derivative substance of clofibrate, designed to improve lipid metabolism in human. CA, is a bioactive metabolite of serum triglyceride-lowering pharmaceuticals (ethofyllinclofibrate, etofibrate and clofibrate), used as antilipaemic agents (Kosjek et al., 2009) and also considered as a potential endocrine disruptor, because it interferes with the synthesis of cholesterol (Pfluger and Dietrich, 2001). CA is persistent for a long time (e.g., approximately 21 years) in the environment (Daughton and Ternes, 1999; Doll and Frimmel, 2003; Gros et al., 2006).

Occurrence of CA in the environment has been reported in many countries. For example, 1 ng/L in the North Sea (Weigel et al., 2002); 103 ng/L in Detroit River water (Boyd et al., 2003); 270 ng/L in drinking water of Berlin area (Tauxe-Wuersch et al., 2005) and 0.55\(\mu\)g/L in surface waters of Swiss lakes (Buser et al., 1998). Moreover, CA was also detected in effluents from sewage treatment plants at 1.6 \(\mu\)g/L in Germany (Ternes, 1998), 0.8 - 2 \(\mu\)g/L in USA (Hignite and Azarnoff, 1977) and 5 ng/L in Greece (Koutsouba et al., 2003). Because of its polar character, clofibric acid does not significantly adsorb in soil and can easily spread in surface and groundwater (Rosal et al., 2009).

The toxicity of lipid lowering agents particularly, CA is not extensively studied. Clofibrate showed mean lethal concentration (LC50) values in the range of 7.7 to 39.7 mg/L and also at 87.22-526.5 mg/L in different organisms. The fish \textit{Gambusia holbrooki} exposed to LC50 value of 7.7 mg/L for 96 h seems to be most sensitive to acute clofibrate concentrations that has been studied (Nunes et al., 2004).
Significant dose-related increases in peroxisomal FOA were observed after clofibrate showed a no observed effect concentration (NOECs) for reproduction in *Daphnia* at 10 μg/L; NOECs for CA in *C. dubia* to 7 days (d) (640 μg/L), the rotifer *B. calyciflorus* (NOEC (2 d) = 246 μg/L), and early life stages of zebrafish (10 d) for 70 mg/L were reported (Ferrari *et al*., 2003). Freshwater fish *C. carpio* exposed to various concentrations of CA (1, 10 and 100 μg/L) showed significant alterations on haematological, biochemical, ionoregulatory and enzymological alterations (Saravanan *et al*., 2011b).

The non-steroidal anti-inflammatory drugs (NSAIDs) are among the most used pharmaceuticals in the world (Tauxewuersch *et al*., 2005; Pamplona *et al*., 2011). NSAIDs are among the most abundant environmental pharmaceutical contaminants due to their high volume of consumption (about 50,000 tons a year) and incomplete removal during wastewater treatment (Nakada *et al*., 2006; Bhandari and Venables, 2011). Diclofenac (DCF), one of the most important NSAID and largely used to reduce inflammation, pain in conditions like arthritis, dental surgery and dysmenorrhea, migraine, menstrual, dysmenorrheal and rheumatic disease and also act as a cyclooxygenase inhibitor (Alves and Wood, 2006; Kosjek *et al*., 2009) and 15% is excreted unchanged after human consumption (Landsdorp *et al*., 1990).

DCF also detected extensively in different water bodies throughout the world because of its higher amount of usage and large amount of production. It is one of the most frequently detected pharmaceuticals in waters and urban wastewaters (Andreozzi *et al*., 2003), since its biodegradation in wastewater treatment plants is limited (Tauxe-Wuersch *et al*., 2005). In this regard, there are many reports available like 10-29 μg/L in the Lake Greifensee (Buser *et al*., 1998), 1030 ng/L (Heberer *et al*., 2002) and 15 μg/L in surface waters (Rabiet *et al*., 2006), 2 ng/L in drinking water well, Mediterranean region (Rabiet *et al*., 2006), 0.38 μg/L in groundwater, Berlin (Jux *et al*., 2002), 195 ng/L in Mersey estuary (UK) (Thomas and Hilton, 2004) and 6.2 ng/L in estuary of the river Elbe, North Sea (Bercu *et al*., 2008). Plant effluents and surface waters have been detected in the range of 0.14-1.48 mg/L (Zhang *et al*., 2008). Lethality and teratogenicity were observed in DCF exposed zebra fish embryos (*Danio rerio*) after 96 h exposure to 480 ± 50 μg/L (LC50/96 h) and 90 ± 20 μg/L (EC50/96 h), respectively (Dietrich and Prietz, 1999). Short-term acute toxicity was analyzed...
in algae and invertebrates (Webb, 2001; Cleuvers 2003). Phytoplankton was more sensitive (lowest EC50 (96 h) = 14.5 mg/L, than zooplankton (lowest EC50 (96 h) = 22.43 mg/L (Ferrari et al., 2004).

According to Petrocelli (1985) an important tool for understanding and evaluating the potential hazard of chemicals to aquatic organisms is the chronic or full life cycle toxicity tests; a chronic toxicity test can indicate concentration of a chemical that will interfere with normal growth, development and attainment of reproductive potential of an aquatic organism; concentrations that produce chronic effects are lower than those produce readily observable acute effects such as mortality; therefore, chronic toxicity test can provide a more sensitive measure of chemical toxicity than acute toxicity tests. Due to continuous entry of pharmaceuticals into aquatic environments, it is necessary to perform chronic studies to observe the long-term effects of drugs on non-target organisms (Daughton and Ternes, 1999; Fent et al., 2006). Further, biomonitoring using chronic toxicity assay may sensitively indicate the pollution stress posed by the pollutants (Zhou et al., 2008).

In assessment of polluted water bodies and aquatic animal health biomarkers are widely used as early diagnostic tools (Cajaraville et al., 2000; Berninger and Brooks, 2010). Biomarkers are biological responses that can be specified in terms of a molecular or cellular event and are measurable with precision and yield reliable information on either the degree of exposure to a chemical and/or its effect upon the organisms or both. In this case, blood is used as a patho-physiological reflector of the whole body, so blood parameters are important in diagnosing the structural and functional status of the animal exposed to the toxicant. Changes in blood parameters are often quick to environmental or physiological alterations, further more they are easily measurable and provide an integrated measure of the physiological status of organisms. Biochemical, physiological, blood chemistry, ionoregulatory and enzymological parameters have been routinely used as valuable biomarkers to assess the toxicity of any environmental contaminants on aquatic ecosystem (Li et al., 2010, Saravanan et al., 2011; Saravanan et al., 2011b).

Environmental contaminants generate serious problems to the aquatic organism, especially on disturbing thyroid hormone homeostasis in species
(Boas et al., 2006; Miller et al., 2009). Thus, endocrine responses through their integrative functions and its early warning capacity may offer as potential indicators that may be useful in the detection/assessment of toxic stress in fish exposed to polluted environments (Hontela et al., 1993). And also measurement of circulating levels of hormones can provide additional information on the lethal effects of many chemicals (Folmar, 1993). Ion levels in plasma as measured by osmolality or specific ion concentrations of sodium (Na\(^+\)), potassium (K\(^+\)), and chloride (Cl\(^-\)) have potential as sensitive biomarkers of pharmaceutical exposure (Saravanan et al., 2011b). Biochemical parameters like plasma glucose and protein are widely used to assess the toxic stress induced by pharmaceutical drugs (Li et al., 2011; Saravanan et al., 2011a). Enzyme activities have also been used as sensitive indicator of stress in fish exposed to diverse group of water pollutants and also to predict the possible level of threat to life (Kavitha et al., 2010; Saravanan et al., 2011b). In addition, gill Na\(^+\)/K\(^+\)-ATPase is involved in osmoregulation of fish and widely used as a sensitive indicator of environmental contaminants (Suvetha et al., 2010; Saravanan et al., 2011b).

Embry et al. (2010) reported that aquatic toxicity data on acute and chronic responses to anthropogenic chemicals by fish plays a very important role. As a result, evaluation of the toxicological and ecological relevance of drug concentrations measured in the environment is still problematic. In contrast, effect data for the acute and chronic toxicity of human pharmaceuticals in wildlife are still scarce (Triebskorn et al., 2007). Currently, there is a need for more specific toxicological investigations focusing on specific targets of the pharmaceutical in lower vertebrates and invertebrates. This is based on the hypothesis that the modes of action are similar as in humans. Mass fluxes alone are insufficient to evaluate the risk stemming from pharmaceuticals; their ecotoxic potential needs to be considered, what to our knowledge has not been done so far.

Although several residual pharmaceuticals are unlikely to result in lethal toxicity in aquatic organisms because of low concentrations combined with low toxicity, prolonged exposure may lead to observable toxic effects (Li et al., 2011). In this case, only little is known about ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms and wildlife, and thus a comprehensive review on
ecotoxicological effects is lacking. Moreover, the knowledge on the toxicity and effects of pharmaceutical drugs on the aquatic organisms are meager, particularly on freshwater fish.

The adverse effects of toxicants become significant when they affect economically important organisms or affect those organisms which are consumed by economically important animals and human beings and it produces stress conditions either in the form of physiological, biochemical, damage to vital organs or even death of living organisms of terrestrial and aquatic environment (Kumar et al., 2011). However, most investigations have been limited to lethal effects during acute and chronic exposures (Han et al., 2010) and the majority of published data is based on far higher concentrations than those detected in freshwater systems. In this study, the present knowledge about acute and chronic effects of human pharmaceuticals (some of which are also applied in veterinary medicine) on aquatic organisms is critically reviewed.

Studies considering the implications of drugs on aquatic organisms at environmentally realistic concentrations are required (Dietrich et al., 2010a). Further, there is a lack of available information about the toxic effects of CA and DCF in the aquatic environment, thus it is important to investigate its potential ecotoxicity. To our knowledge, the toxicity of CA and DCF has been studied a little. Hence, the present study is planned to examine the impacts of most commonly used and detected pharmaceuticals CA and DCF in an Indian major carp, *C. mrigala* with the following objectives.

- To investigate the toxicity of CA and DCF on *C. mrigala* under short-term (96 h) and long-term exposures (35 days) at different concentrations viz., 1, 10 and 100 µg/L.
- To estimate the hormonal responses (TSH, T\(_4\), and T\(_3\)) in the plasma of *C. mrigala* treated with various concentrations of CA and DCF under short and long term exposures and to use thyroid endpoints as biomarkers of exposure to environmental pollutants
• These hormones play an important role in the maintenance of a normal physiological state in vertebrates, and also being important targets of xenobiotics.

• To analyze ionoregulatory (Na\(^+\), K\(^+\) and Cl\(^-\)) disturbances in the plasma of fish treated with various concentrations of CA and DCF under short and long term exposures and to use these parameters as sensitive biomarkers of pharmaceutical exposure.

• To observe the glucose and protein contents in plasma of fish exposed to different concentrations of CA and DCF at short and long term exposures and to use these parameters as an important biomarkers in the field of environmental biomonitering.

• To study the enzyme activity (GOT and GPT) in plasma and Na\(^+\)/K\(^+\)-ATPase activity in gill of the fish exposed to different concentrations of CA and DCF under short and long term exposures and to use their level as an indicator for tissue damage and cell rupture.