EVALUATION OF THE EFFECT OF AN ENDOCRINE DISRUPTING CHEMICAL, CHLORDECONE, IN THE REPRODUCTIVE AND NON-REPRODUCTIVE TISSUES OF THE CICHLID FISH, *PSEUDETROPLUS MACULATUS* (BLOCH, 1795)

Thesis submitted to the
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DOCTOR OF PHILOSOPHY IN ZOOLOGY
Under the Faculty of Sciences

BY

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CERTIFICATE

This is to certify that Ms. K.P. Asifa has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph. D ordinance of this University. I recommend her thesis entitled "Evaluation of the effect of an endocrine disrupting chemical, chlordecone, in the reproductive and non-reproductive tissues of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795)" for submission for the degree of Doctor of Philosophy in this University.

I further certify that this thesis represents the independent work of the candidate under my supervision and no part of the thesis has been presented for the award of any other degree, diploma or associateship in any University.

Dr. K. C. Chitra
Supervisor

DECLARATION

I hereby declare that the work presented in the thesis entitled "**Evaluation of the effect of an endocrine disrupting chemical, chlordecone, in the reproductive and non-reproductive tissues of the cichlid fish, *Pseudetroplus maculatus* (Bloch, 1795)**" is a genuine record of research work done carried out by me under the guidance and supervision of Dr. K.C. Chitra, Assistant Professor, Department of Zoology, University of Calicut.

To the best of my knowledge, no part of this thesis has been previously submitted for the award of any degree, diploma or associateship in any other University.

(Asifa. K. P.)
Candidate

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19 December, 2017.

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Asifa. K. P

DEDICATED

TO

MY FAMILY

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
ADB	Asian Development Bank
AHH	Aryl Hydrocarbon Hydroxylase
AHR	Aryl Hydrocarbon Receptor
AhRE	Aryl Hydrocarbon Responsive Element
ALP	Alkali-Labile Phosphoprotein
ALKP	Alkaline Phosphatase
ANOVA	Analysis of Variance
APHA	American Public Health Association
AR	Androgen Receptor
ARNT	Aryl Receptor Nuclear Translocase
ATP	Adenosine Tri Phosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BCKDH	Branched-Chain Alpha-Keto Acid Dehydrogenase
BSA	Bovine Serum Albumin
˚C	Degree Celsius
CAT	Catalase
COX	Cyclo-Oxygenases
CYPscc	Cytochrome P450-side chain cleavage enzyme
DHP	Dihydroxy-4-pregnen-3-one
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPX	Distyrene Plasticized Xylene
E2	Estradiol
EC_{50}	Median effective concentration
ED_{50}	Median Effective Dose
EDC	Endocrine Disrupting Chemical
ELISA	Enzyme Linked Immuno Sorbent Assay
ER	Estrogen Receptor
EROD	7-EthoxyResorufin-*O*-Deethylase
ETC	Electron Transport Chain
ETFQO	Electron-Transfer Flavoprotein - Ubiquinone Oxidoreductase
FAD	Flavin Adenine Dinucleotide
FMO	Flavin Monooxigenase
FSH	Follicle-Stimulating Hormone
FSH-R	Follicle-Stimulating Hormone Receptor
g	Acceleration due to Gravity
g	Gram
GnRH	Gonadotropin-Releasing Hormone
GPCR	G-Protein Coupled Receptor
GPx	Glutathione Peroxidase
GR	Glutathione Reductase

LIST OF ABBREVIATIONS (Continued..)

GSH	Reduced Glutathione
GSI	Gonadosomatic index
GSSG	Oxidised Glutathione
GST	Glutathione-S-Transferase
GTH	Gonadotropic Hormone
h	Hour
HCB	Hexachlorobenzene
HSI	Hepatosomatic index
HPG	Hypothalamic-Pituitary-Gonadal axis
HSD	HydroxySteroid Dehydrogenase
HSDB	Hazardous Substances Data Bank
hsp	Heat Shock Protein
kDa	Kilo Dalton
kg	Kilo gram
KT	Ketotestosterone
L	Litre
LC	Lethal Concentration
LC_{50}	Median Lethal Concentration
LD	Lethal Dose
LD_{50}	Median Lethal Dose
LH	Luteinizing Hormone
LH-R	Luteinizing Hormone Receptor
LOX	Lipoxygenases
MDA	Malondialdehyde
mg	Milligram
min	Minute
MIS	Maturation Inducing Steroid
mM	MilliMolar
Mn-SOD	Mitochondrial Superoxide Dismutase
MRP	Multidrug Resistance Proteins
NAD	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide phosphate
NAT	n-Acetyltransferase
NIOSH	National Institute for Occupational Safety and Health
nm	Nanometer
NR	Nuclear Receptor
ODH	Oxoglutarate Dehydrogenase
OECD	Organization for Economic Co-operation and Development
P	Probability
PDH	Pyruvate Dehydrogenase
Pgp	P-glycoprotein
PPAR	Peroxisome Proliferator-Activated Receptors
ppt	parts per trillion
PUFA	Polyunsaturated Fatty Acids

LIST OF ABBREVIATIONS (Continued..)

Q	Quinol
r	Correlation Co-efficient
RBC	Red Blood Corpuscles
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RXR	Retinoid X Receptor
SD	Standard Deviation
SDH	Succinate Dehydrogenase
sn-G3PDH	Sn-Glycerol-3-Phosphate Dehydrogenase
SOD	Superoxide Dismutase
SPSS	Statistical Package for the Social Sciences
SQR	Succinate-Coenzyme Q Reductase
StAR	Steroidogenic Acute Regulatory protein
SULT	Sulphotransferase
T	Testosterone
TBARS	Thiobarbituric Acid-Reactive Substances
TR	Thyroid Hormone Receptor
TSH	Thyroid Stimulating Hormone
UGT	UDP-glucuronosyltransferase
UN	United Nations
UNEP	United Nations Environment Programme
v	Volume
Vtg	Vitellogenin
vtgR	Vitellogenin Receptor
w	Weight
WBC	White Blood Corpuscles
WHO	World Health Organization
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
γ-GTP	Gamma glutamyltranspeptidase
µg	Microgram

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CHAPTER 1

Evaluation of acute chlordecone toxicity in the fish, *Pseudetroplus maculatus*

General Introduction

Aquatic toxicology is the study of the effects of environmental contaminants such as pesticides, metals, nanomaterials, phthalate plasticizers etc. on the health status of aquatic organisms, particularly on fish and crustacean. Recently, there is a great concern regarding the adverse effects caused by the exposure to environmental contaminants. Rapid increase in industrialization, urbanization and agricultural productivity are the main reasons of the continuous release of hazardous contaminants into the aquatic ecosystem. Among several environmental contaminants, pesticides are the only group of chemicals that are intentionally used in large-scale to eradicate pests and to protect the agricultural and industrial products. But excessive use of pesticides damages not only the environment and agriculture, through the food chain it contributes to the pollution of aquatic ecosystem and also affects the health status of aquatic fauna. Aquatic resources such as ponds, rivers, lakes, streams, oceans and of course fisheries are considered as remarkably valuable natural assets to humans worldwide. Therefore, release of toxicants that affect the aquatic life and water quality has increased concern on the growing human population. Moreover, it is evident that the surplus use of pesticides is one of the major factors that lead to the decline in fish population (Relyea and Hoverman, 2008).

The effects of pesticides that harm the aquatic organisms depends on several aspects such as nature of the toxicant, dosage rate, time of exposure and the mode of persistence in the environment. Most of the toxicants lack target specificity, and elicit severe toxic effects on non-target aquatic species including bacteria and other invertebrates apart from the fish population. Fish and other aquatic animals are therefore exposed to toxicants depending on several factors namely bioavailability, bioconcentration, biomagnification and persistence in the environment (Alexander, 1999). The concentration or dose of the toxicants that are available naturally in the environment i.e., environmental concentration, to which fish or any aquatic animals exposed is called bioavailability of the toxicant. Some toxicants readily breakdown and make themselves available in the environment, whereas some tightly bind to soil or any suspended particles in aquatic environment thereby reduce its availability. While some toxicants are converted into less toxic metabolites or quickly diluted in

water or vaporized into the air thereby making less available to aquatic animals (Gourlay-France and Tusseau-Vuillemin, 2013).

The accumulation of toxicants in tissues of exposed animals at greater level than it was found in water or soil to which the contaminants were applied is called bioconcentration. Some of the fish or other aquatic animals concentrate the toxicants in body tissues or organs at about 10 million times more than in the environmental concentration. Owing to the lipophilic properties, generally pollutants in water are readily absorbed into the body of animals and cause bioaccumulation, which in turn augment through the food chain by biomagnification. Thus organisms at the bottom of the food chain absorb the toxicant from the water and accumulate in its various tissues. Organisms at the next tropic level, especially fishes, while feeding them receive a higher dose of the chemical and further accumulation occurs in the tissues. Thus, organisms at the top of the food chain, especially humans, are known to expose to the toxicants at a much higher level than present in the water indirectly and could cause severe health problems (Chopra *et al.,* 2011).

The toxicity of pollutants also depends on the length of time up to which the toxicants remain as such in the environment and is often called persistence. Toxicants can be easily degraded or break down into less toxic substances either by exposure to sunlight – photodegradation, microbial decomposition, soil condition etc. Toxicants are said to be persistent when it breakdown slowly so that it is more available as parent compound to aquatic animals. Toxicants are less persistent when rapidly breakdown into less toxic metabolites or disappears from the exposed environment. It is usually expressed as half-life of the toxicant, which is nothing but the time required by the toxicant to degrade or disappear to half of the amount. Therefore, the severity of health effects of aquatic animals exposed to toxicants depends on several factors as mentioned. Even unintentional release of environmental contaminants as pesticides have great impact on the ecosystem as well as it adversely affect the fitness of aquatic life which ultimately leads to decline in fish population. Thus aquatic pollution is a major cause of global concern as it leads to degradation of aquatic biodiversity together with development of various health problems such as fatal diseases in higher organisms, including humans. The

overall consequences of toxicants can be minimized only when its usage and application is reduced globally in non-target areas. The adverse global effects of any anthropogenic or natural toxicants on aquatic organisms at various levels as subcellular, tissue specific, individual organisms, communities or ecosystems is documented through aquatic ecotoxicology.

1.1 Environmental contaminants

Environmental contaminants are chemicals which are introduced into the environment accidentally or intentionally that may have the potential to harm humans, wildlife, aquatic animals and plants. Numerous harmful chemicals are known to contaminate water, land, or air every day and lead to severe health effects in exposed organisms and also influence the quality of the environment. Water pollution is one of the most serious ecological threats faced in recent days. Natural water resources are being squeezed by human activities like discharge of untreated industrial and sewage wastes and agricultural runoff. This has frequently resulted in conversion of rivers, ponds, lakes and coastal waters into sewage depots where the natural biological equilibrium is severely upset. Exposures to environmental pollutants hang about a major source of health risk throughout the world and usually the threats are higher in developing countries because of weak environmental legislation, unplanned industrial growth, use of outdated technologies, lack of policies to control pollution and inefficient waste disposal together roots for the rise in pollution level (Firdaus and Ahmad, 2010).

Natural calamities such as storm drains, volcanoes, mining, algal blooms and earthquakes have been shown to cause major changes in the quality and ecological status of aquatic ecosystem. The toxicity of environmental contaminant mainly depends on the concentration of toxicant that reaches the organism or the affinity of target receptor in the organism. The absorption of toxicant by the organism is based on the structure of toxicant, nature of solubility in water or fat, characteristics of the external covering of organisms, feeding behavior, and perception of other vital systems in the body of animals (Landrum and Fisher, 1999). Thus it is evident that the mode of action of contaminants varies among different organisms depending on

the physico-chemical properties of the exposed compound as well as physiological characteristics of the organisms exposed.

Despite the properties of the contaminants, the dose, duration, route of exposure, species exposed, developmental stages of animals at which the contaminant exposed are the other basic features for the toxicity of contaminants. The contaminants even at sublethal concentrations may interfere with several physiological processes along with behavioural alterations in fishes and other aquatic organisms without causing death, but could affect the survival of a species (Heath, 1995; Scott and Sloman, 2004). The pollutants enter into the body of fish and other aquatic animals living in the contaminated ecosystem through multiple routes. The mode of entry occur mainly through direct absorption through the skin, uptake of contaminated water through the gills and mouth at the times of respiration and drinking as well as feeding the toxicant contaminated prey. Alterations in the chemical composition of natural aquatic environments by the pollutants generally induce changes in the behaviour and physiology of the exposed fish. This continuous and distressing influx of toxicants into the aquatic environments may cause demolition of beneficial species either directly affecting their life or indirectly through violating biological food chains. Even though most of these compounds are present at low concentrations, many of them raise substantial toxicological concerns, mostly when present as components of complex mixtures (Helfrich *et al*., 2009).

Important groups of environmental contaminants include pharmaceuticals, synthetic hormones, metals, polyethoxylates, pesticides, herbicides, fungicides, antibacterial agents, plasticizers, personnel care products, polychlorinated biphenyls, phthalates, dioxins, polychlorinated aromatic hydrocarbons and polybrominated diphenyl esters (Riley and Zachara, 1992; Kolpin *et al.*, 2002; Boxall *et al.*, 2012). Among this, some of the contaminants elicit their toxic effects by altering the normal functions of the endocrine system are called endocrine disrupting chemicals (EDCs) or xenoestrogens. Although limited information is available on the potential adverse effects of xenoestrogens in aquatic life, there is an increasing concern on the very low levels of EDCs could have adverse effects in fish reproduction thereby resulting in reduced fertility and decline in the fish population.

1.2 Endocrine disrupting chemicals - Xenoestrogens

"Endocrine disrupting chemicals (EDCs) are exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Diamanti-Kandarakis *et al.*, 2009, p 294). These chemicals are also known in different names such as hormonally active agents, endocrine modulators, endocrine disruptors, xenohormones, environmental hormones, xenoestrogens or endocrine active compounds. It is now being recognized that numerous endocrine disruptors have been released into the environment in large quantities since World War II. Several environmental contaminants even at very low concentrations disturb the endocrine system and modify the secretion of various hormones causing permanent and irreversible effects in the exposed organisms. Endocrine disruptors not only affect the endocrine system it also have the potential of bioaccumulation and biomagnification in various tissues (Langston *et al.*, 2005).

Endocrine disruptors also known to affect at every level of endocrine function either by disrupting the enzymes involved in steroidogenesis or by negatively interacting with the transport of hormones in target sites (Déchaud *et al.*, 1999). EDCs are known to restrain several physiological processes in animals through the disruption of various hormones associated with it. Sex steroids are important targets of most of the EDCs, which in turn induce several reproductive and developmental anomalies such as development and functioning of gonads, gametogenesis, fertilization and embryogenesis in fishes and other vertebrates including humans (Guillette *et al.*, 1995; Norris and Carr, 2005; Scholz and Kluver, 2009; Ciocan *et al.*, 2010). Generally, EDCs exert their actions primarily through members of the family of nuclear receptors (NRs), especially estrogen receptors (ERs) and androgen receptor (ARs). Additionally, the thyroid hormone receptors (TRs), retinoid X receptor (RXR), aryl hydrocarbon receptor and peroxisome proliferator-activated receptors (PPARs) also mediate the action of EDCs (Swedenborg *et al.*, 2009). EDCs also elicit the effects by modulating co-activators of nuclear receptor, proteasome-mediated degradation of nuclear receptors, hormone

sensitizers and DNA methylation (Tabb and Blumberg, 2006). Therefore, EDCs are known to induce conformational changes in the tertiary structure of ERs so that the recruitment of cofactors is highly affected (Déchaud *et al*., 1999). Interactions between ERs and cofactors or co-repressors are important to regulate ER-mediated transcription and for the modulation of the expression of ER-target genes (Yang *et al*., 2015).

Most of the EDCs in the environment interfere with reproduction of animals by acting as either agonists or antagonists of the sex steroidal hormones, estrogens or androgens, and alter the normal levels of reproductive hormones (Sonnenschein and Soto, 1998). As EDCs possess the properties of natural endogenous estrogens it could cause alterations in the levels of hormones through changes in the production, transport or metabolism. It ultimately lead to drastic sexual and reproductive abnormalities in animals, including fishes causing feminization in male (Tyler and Jobling, 2008), formation of intersex population (Bahamonde *et al.*, 2013), induction of vitellogenin (Arukwe and Goksoyr, 2003), reproductive tract malformations (Nikaido *et al.*, 2004) and cancer in liver, breast, testis and ovary (Toppari *et al.*, 1996; Fernandez *et al.*, 2004; Park *et al.*, 2009). Generally xenoestrogens are less potent than endogenous estrogens when tested in bioassays, but certain properties such as resistance to chemical or enzymatic degradation, persistence in the environment, lipophilicity, storage and sequestration by adipose tissue, and distorted excretion by the organism generate a cause for concern (Korach, 1993; Stone, 1994).

EDCs includes several classes of chemicals such as polychlorinated biphenyles, dioxins, pharmaceuticals, phytohormones, detergents, pesticides, plastics, plasticizers, fuels, and many other chemicals that are present in the environment. These contaminants are continuously released in the environment due to the widespread and diverse applications such as manufacturing of plastics, fuels, pesticides, flame retardants in different products, sewage treatment plants, drugs, cosmetics and personnel care products etc. (Li *et al.*, 1985; Krishnan *et al.*, 1993; White *et al.*, 1994; Kojima *et al.*, 2004). Among the different class of EDCs, organochlorine pesticides contribute a major source of contamination in the aquatic environment.

1.3 Organochlorine pesticides

Pesticides have long been used in agriculture to control pests and that ensure increased food production. However, World Health Organization (WHO) reported that roughly three million pesticide poisoning occurs annually which results in 220,000 deaths worldwide (WHO, 1992). There are enormous literatures stating the increased incidence of disease such as allergies, neurological and reproductive disorders and even the augmented frequency of cancer across the world may be related to the exposure of pesticides. The major route of pesticide exposure to aquatic ecosystem is through accidental spillage, rainfall runoff and from industrial and municipal effluents (Baker and Richards, 1990). The uncontrolled and inappropriate applications of pesticides have raised serious concerns about various ecosystems in general and the health of humans, wildlife, fishes and animals in particular. Pesticide application potentially affect non-target species in aquatic ecosystem, particularly fishes are very sensitive to the change in the quality of water. The contamination of water bodies by pesticides is associated with intense toxic effects of growth, survival and reproduction of aquatic organisms. Therefore, exposure to pesticides could alter normal physiological and biochemical process in vital organs of fishes (Agrawal *et al.*, 2010).

Fish absorbs the toxicants exposed through skin, gill or gastrointestinal tract. Most of the pesticides are lipophilic showing affinity to biological membranes and increases sensitivity to exposed pesticides. Toxic effects of several pesticides in different fishes has been reported globally causing a wide range of effects including behavioral modifications, oxidative damage, inhibition of acetylcholinesterase activity, developmental changes, endocrine disruption, histopathological alterations, genotoxicity and carcinogenicity (Murthy *et al.*, 2013). Moreover, pesticides can indirectly enter the body of higher organisms including humans through the food chain because most of the pesticides have the potential of bioconcentration and biomagnification. However, detoxification and elimination of toxicants are facilitated by well developed detoxification mechanisms including enzymatic biotransformation.

Among pesticides, organochlorine pesticides are most extensively studied due to their widespread application and detrimental effects on both terrestrial and aquatic animals. Organochlorine pesticides are a diverse group of persistent, synthetic chlorinated hydrocarbons widely used for the control of various classes of agricultural pests possessing carcinogenic and endocrine disrupting properties. In Asia, the use of pesticides, particularly organochlorine pesticides is about 40% of the total when compared to the other developing countries (Gupta, 2004). Organochlorine pesticides are lipophilic which can easily cross the lipid bilayer membrane and get widely distributed throughout the body. Metabolic conversion of lipophilic compound into hydro-soluble compounds is very necessary before being excreted from the body of the animal. Usually the biotransformation of organochlorine pesticides occur so slowly and it would result in several adverse modifications within the animal that ultimately kill the organism itself (Parkinson, 2001). Organochlorine toxicity is mainly due to stimulation of central nervous system.

Several literatures have demonstrated different toxicological endpoints in fishes and other animals including humans. Toxic effects of pesticides to humans is usually assessed by measuring the levels of pesticides in human samples such as breast milk, maternal blood and serum, sperm samples, urine, umbilical cord blood etc. (Kaiser, 2000). In fishes, the most apparent symptoms of organochlorine pesticides include lethargy, mucous secretion, aggressive swimming, other neurological disorder, disruption of nerve functions, respiratory dysfunction and suffocation (Banaee *et al*., 2011). In addition, the acute toxicity of the pesticides also depends on various factors as age, size and sex of fish, time of exposure, purity and formulation of pesticides. Available data revealed that one of the organochlorine pesticides, chlordecone, that possess wide application globally cancelled the registration in 1978 throughout the world except in India. The use of chlordecone is more predominant in India and it is widely available in different names across the country. It was primarily used as an insecticide and peculiar property of chlordecone is its resistance to degradation in the environment and its potential for

bioaccumulation in fish and other aquatic organisms (Agency for Toxic Substances and Disease Registry, ATSDR, 1995).

1.4 Chlordecone

Chlordecone is a synthetic, chlorinated, polycyclic, ketonic pesticide which is also known by its trade name Kepone®. It was produced in the United States in early 1950s and commercially introduced in 1958 (Huff and Gerstner, 1978). Chlordecone was primarily used as an insecticide under the trade names Kepone, Mirex, Kelevan and Curlone. There was a negative impact regarding chlordecone on the public, environmental and occupational health. As a result of negative human health impact of chlordecone, the production, sale and use of chlordecone was prohibited in all developed countries. However, no such regulations or ban of chlordecone was established in India and it is used widely as pesticide throughout all states of the country, including Kerala in the name of Kelevan®.

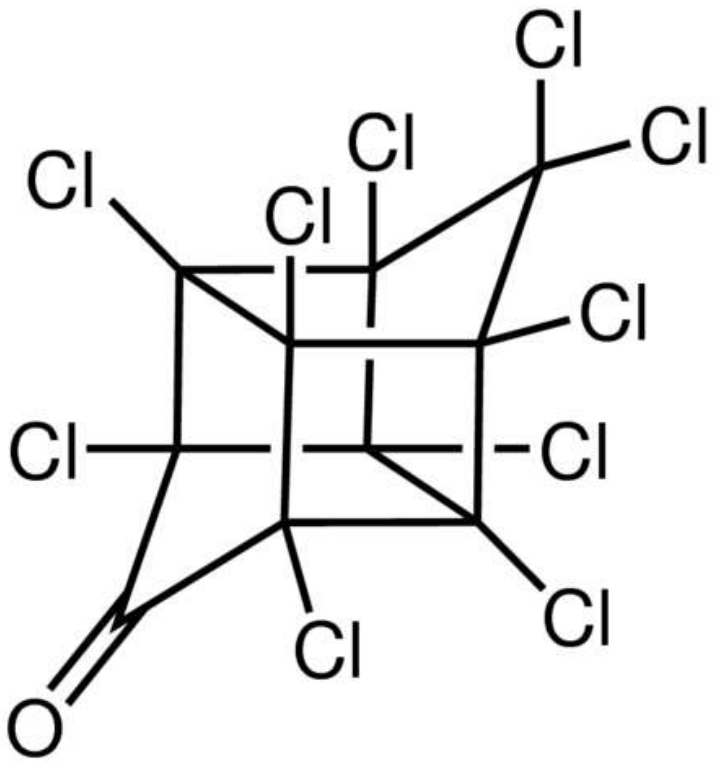
Chlordecone is a stable compound and has been shown to have high resistance to physical and biological degradation where it remains in the environment for a long period of time (Carlson *et al.*, 1976). Within the soil, it binds to soil particles and after rainy episodes, rain water wash out chlordecone from soil, to the surface waters where it becomes a source of contamination for aquatic organisms. It has been reported that the half-life of chlordecone in river is 3.8 to 46 years (HSBD, 2009). It does not dissolve easily in water, but preferentially bind to soil and sediment particles (Huggett and Bender, 1980). As a result chlordecone stay for several years in soil, water and sediments and cause harmful effects on aquatic life, particularly fish and finally to human health through fish food consumption.

1.4.1 Properties of chlordecone

Chemical name	:	decachlorooctahydro-1,3,4-metheno-2H-cyclobuta[cd]-pentalen-2-one
Chemical formula	:	$C_{10}Cl_{10}O$
Molar mass	:	490.633 g/mol
CAS number	:	143-50-0

Appearance	:	tan to white crystalline solid
Odour	:	Odourless
Density	:	1.6 g/ cm^3
Melting point	:	349ºC (660ºF; 622 K)
Solubility in water	:	2.7 mg/ L at 25ºC
Solubility:		acetone, hexane, dimethylsulfoxide, ketone, acetic acid, alcohol
Vapour pressure	:	2.25 X 10^{-7} mm Hg at 25ºC
Density	:	1.61 g/ mL at 25ºC
Partition co-efficient	:	log kow = 5.41

(Wikipedia – IUPAC information)



Structure of chlordecone

1.4.2 Uses

The synthetic organochlorine pesticide, chlordecone, is widely used as an agricultural insecticide, miticide and fungicide. Chlordecone has been extensively used for the control of banana root borer, rust mites on non-fruit bearing citrus trees,

wire worms on tobacco fields as well as control of apple scab and powdery mildew, grass mole cricket, slugs, snails, and fire ants in ornamental shrubs. Chlordecone is also used in the household products such as ant and roach traps (ATSDR, 1995).

1.5 Effects of chlordecone in humans

In 1950s the production of chlordecone was first patented by Allied Chemicals Corporation, Hopewell, USA. The waste materials released as a result of the production were not properly disposed and were directly released into the nearby River James. Thus fish or animals that live in aquatic ecosystem near the production area were highly exposed to chlordecone. The people lived nearby the site of production experienced several health problems due to the toxicity of chlordecone. Several studies revealed that chlordecone can pass through skin into the bloodstream where it enters into the lungs through polluted air and also found in saliva and human milk (Cannon *et al*., 1978). From the bloodstream, chlordecone is transferred by means of secretory process to gastrointestinal lumen that is governed by simple diffusion (Bungay *et al*., 1979). Workers exposed to chlordecone mainly through inhalation and dermal contact showed harmful effects on the nervous system, liver, skin and male reproductive system (Cohn *et al.*, 1978; Taylor, 1982). Employees exposed to chlordecone during its manufacture developed skin rashes, irritability, trembling, headache, blurred vision, enlarged liver and kidney failure (Taylor, 1982).

Chlordecone is possibly carcinogenic to humans that caused liver, adrenal and blood cancer (Guzelian, 1982). Therefore, The National Institute for Occupational Safety and Health (NIOSH) recommended that the average workroom air level of chlordecone should not exceed 50 parts per trillion (ppt) over eight hour (ATSDR, 1995). Chlordecone at moderate amount were noticed in muscle, gall bladder, bile, and stool, while in aqueous body fluids such as cerebrospinal fluid, urine, saliva, and gastric juice only trace amounts were observed (Cohn *et al*., 1978). Chlordecone is an estrogenic endocrine disrupting chemical causing alterations in blood concentrations of sex steroid hormones in humans (Hammond *et al*., 1979). In humans, elimination of chlordecone from the blood is very slow and the serum half-

life ($t_{1/2}$) estimated in chemical plant workers ranged from 63 to 128 days (Adir *et al*., 1978).

1.6 Effects of chlordecone in vertebrates

Percutaneous absorption and disposition of [14C]chlordecone in young and adult female rats showed only a limited absorption (Heatherington *et al.,* 1998). Chlordecone exposed to isolated perfused pig liver showed an increase in high density lipoprotein which may affect the distribution of chlordecone, favouring chlordecone uptake and retention in the liver and decreased chlordecone elimination in the bile (Soine *et al*., 1984). In rat, treatment of chlordecone at a single oral dose of 50 mg/ kg showed highest tissue concentrations in liver followed by plasma, kidney and adipose tissue (Hewitt *et al*., 1985). Chlordecone has been shown to cross the placenta which was observed in rat fetal tissues as early as 4 hours after maternal dosing (Kavlock *et al.,* 1980). Chlordecone also enter into the body of young ones of rat through lactation (Eroschenko and Osman, 1986). *In vivo* studies conducted in rats reported that binding affinity of chlordecone to uterine estrogen receptors is more than that of brain tissue (Williams *et al.,* 1989). The exact reason for *in vivo* tissue-specific difference is not clear, but it may be due to the higher affinity of chlordecone to uterine estrogen receptors and also chlordecone require greater time to reach brain that could result in a less significant estrogenic effect. In contrary, some studies have shown that chlordecone appears to function as an estrogen antagonist in central nervous tissue (Huang and Nelson, 1986).

The neurotoxic effects of chlordecone have been reported in mouse following oral administration at 10, 25 and 50 mg/kg/day and the symptoms include hyperexcitability and tremors on the day 1, 4 and 9 after the toxicant exposure (Huang *et al.,* 1981). Chlordecone produces developmental toxicity in rats and mice at very low concentrations. Neonatal administration of chlordecone in female mice showed similar development changes as induced by estradiol thereby producing morphological alterations in the epithelial lining of the uterus and vagina. At higher concentration of chlordecone the vaginal epithelium experienced keratinization along with hyperplasia, cellular hypertrophy and glandular formation in the uterine

epithelium in dose-dependent manner (Eroschenko and Mousa, 1979). Chlordecone treatment induced fetal toxicity in rat and mice which are evidenced by reduced fetal weight, low degree of ossification, edema, undescended testis, enlarged renal pelvis and cerebral ventricles (Chernoff and Rogers, 1976).

1.7 Effects of chlordecone in aquatic animals

In order to evaluate the toxicity of chlordecone, several acute and chronic studies have been conducted in different aquatic organisms. Chlordecone decreased the concentrations of biomolecules such as protein, ribonucleic acid, deoxyribonucleic acid, adenosine diphosphate and adenosine triphosphate in *Daphnia magna* (McKee and Knowles, 1986). Among aquatic organisms, crustaceans are more sensitive to chlordecone (UNEP, 2007). Studies have shown that chlordecone bioaccumulates in oysters, mysids and grass shrimp where the degree of bioconcentration varies depending on the species and the duration of exposure (Bahner *et al.*, 1977). Apart from bioaccumulation, chlordecone interrupted the moulting process by decreasing the concentration of 20-hydroxyecdysone and the chitobiase activity in *Macrobrachium rosenbergii* (Lafontaine *et al.*, 2016a). Despite the crustaceans, chlordecone is also known to bioaccumulate in fish (Carver and Griffith 1979). Chronic toxicity testing in fathead minnows revealed that the maximum acceptable concentration of chlordecone was between 1.2 and 3.1 μg/L with accumulation in various tissues (Buckler *et al.*, 1981). In sheepshead minnow, exposure of chlordecone caused reduction in fecundity and fertility of eggs, reduced survival of embryos and also resulted in fatty degeneration of liver (Goodman *et al*., 1982).

Chlordecone treatment induced histopathological changes in the liver and gonads of *Heteropneustes fossilis* (Srivastava and Srivastava, 1994a,b). Chlordecone also been reported to alter hematological parameters as total RBC and WBC counts, percentage of hemoglobin, hematocrit and clotting time (Srivastava and Srivastava, 1994). In the catfish, chlordecone induced alteration in carbohydrate metabolism by changing the levels of blood glucose, and liver and muscle glycogen (Srivastava and Srivastava, 1995). Chlordecone showed high affinity to estrogen receptors in both

male and female rare minnow by increasing the level of vitellogenin in females, degenerated gonads and increased the concentrations of plasma estradiol (Yang *et al.*, 2016).

1.8 Mode of action and metabolism of chlordecone

The exact mechanism by which chlordecone is shifted from the gut, lungs, or skin to the blood is not known. However, in contrast with other organochlorine compounds, it is transported through the blood plasma and preferentially distributed in liver tissue rather than the fat tissue. Both *in vitro* and *in vivo* studies on human, rat, and pig plasma showed that chlordecone preferentially bind to albumin and high density lipoprotein and are transported to different tissues (Soine *et al.,* 1982). Since chlordecone is an estrogenic pesticide, it can elicit its effects through binding with estrogen receptors. Studies have shown that following *in vitro* and parenteral exposure, chlordecone bind to estrogen receptors, which cause translocation of the receptor from the cytoplasm to the nuclear fraction (Bulger *et al.,* 1979; Hammond *et al.,* 1979; Williams *et al*., 1989).

Metabolism of chlordecone involves uptake by the liver, enzymatic reduction to chlordecone alcohol, conjugation with glucuronic acid and partial conversion to unidentified polar forms. The final metabolites especially, glucuronide conjugates are then excreted into bile (Fariss *et al.,* 1980; Guzelian *et al.,* 1982). Examination of the bile of occupationally exposed workers revealed that predominant portion was unconjugated (72%), only small portion was conjugated with glucuronic acid or sulphate (9%) whereas the remaining fraction of total chlordecone in bile was stable polar metabolites (19%) which were resistant to β-glucuronidase (Fariss *et al.,* 1980). Thus chlordecone is excreted in human bile as a reduced metabolite, chlordecone alcohol and also in the form of a glucuronide conjugate (Cohn *et al.,* 1978). The hepatic cytosolic aldoketoreductase enzyme called chlordecone reductase catalyses the reduction of chlordecone to chlordecone alcohol and are eliminated from the body primarily through biliary excretion into feces (Molowa *et al.,* 1986a). Whereas, only 5% of chlordecone undergoing biliary excretion was removed in stool daily, which implies it may be subjected to an entero-hepatic cycle for excretion

(Blanke *et al.,* 1978). Fish or other animals that live in water that are contaminated with chlordecone, or on consuming other animals contaminated with chlordecone, can build up the toxic substances in the body, which may be several times greater the amount than in their prey or in the surrounding water (Roberts and Bendl, 1982).

1.9 Fish as an ecotoxicological model

Fish has attracted a considerable attention in recent years as an ecotoxicological model because aquatic ecosystems serve as the final sink for numerous chemicals and, water bodies serves as an ultimate vehicle for the exposure to many toxic contaminants. Fish is a diverse group of aquatic organisms which make them a valuable biological indicator of aquatic environmental pollution and also an excellent laboratory model for the assessment of various contaminants in aquatic ecosystem (Naigaga *et al.,* 2011). When compared with other vertebrates, fishes are more sensitive and it responds with greater susceptibility to numerous toxicants in the aquatic environment. Fishes represent an outstanding laboratory model for aquatic toxicology studies because it can bioaccumulate various toxic contaminants (Streit, 1998). Fish also have well-developed osmoregulatory, endocrine, nervous and immune systems (Song *et al*., 2012). Small size, high fecundity, economical maintenance and use are some of the factors that make fish as a suitable laboratory model. In addition, fish absorb toxicants directly from the surroundings either through dermal contact, or through engulping water containing toxicants it directly enter into the gut or may ingest through contaminated food in the food chain (Suter, 2016). Due to the high mobility, fishes are generally found virtually everywhere in the aquatic ecosystems and occupy the highest position in aquatic food webs. Fish are in the final chain of aquatic food web and a major source of protein-rich food to humans. Therefore, any negative influence on fish population directly reflects the health status of aquatic ecosystem. Water contamination leads to adverse effects on fish which is positively correlated to the human health since fish and fish products are the main protein source of food to humans (Gadzala *et al.,* 2004). Consequently, fish population play a major

ecological role for maintaining aquatic food webs by functioning as a carrier of energy from lower to higher trophic levels (Beyer, 1996).

1.10 *Pseudetroplus maculatus*

The experimental model used in the present study is *Pseudetroplus maculatus*. It is a cichlid fish indigenous to South India and Sri Lanka, which inhabits both freshwater and brackish water habitats (Pethiyagoda *et al.,* 2014). It is widely distributed in almost all rivers, ponds, streams and backwaters of Peninsular India and Sri Lanka (Jayaram, 2010*). P. maculatus* is an omnivorous fish, popularly known as orange chromide. Many variants of *Pseudetroplus* had been identified based on the number of black spots, its arrangements and other characters. Accordingly *Pseudetroplus* is synonymous to *Etroplus* in having eleven pleural ribs instead of 12 to 13 and 26–27 vertebrae instead of 28-29. The anterior half of the median suture between the lower pharyngeal jaws of *Pseudetroplus* is serrated while in *Etroplus* it is smooth. The differences were also noted in the arrangement of anal-fin, haemal spines and in the teeth of the jaws. With reference to the differences in these characters *Pseudetroplus* is being distinguished from *Etroplus* and *Pseudetroplus* is regarded as a synonym of *Etroplus* (Pethiyagoda *et al.*, 2014).

Scientific Classification:

Kingdom	:	Animalia
Phylum	:	Chordata
Subphylum	:	Vertebrata
Class	:	Pisces
Subclass	:	Teleostomi
Super order	:	Actinopterygii
Order	:	Perciformes
Suborder	:	Labroidei
Family	:	Cichlidae
Subfamily	:	Etroplinae
Genus	:	*Pseudetroplus*
Species	:	*maculatus*

P. maculatus is highly nutritious and has high demand as food in India and also available in tropical aquarium as ornamental fish. The gorgeous shape of the body having bright orange colour with black spots, small body size and calm nature attracted the fish of recreational and commercial values. *P. maculatus* is a substrate spawner which exhibit high degree of biparental care on eggs and larvae. Peculiarity in breeding behaviour is also observed in the laboratory conditions. It includes pair formation, nesting at the crevices of pebbles in tanks, biparental caring and spawning intensity made them a successive species in laboratory condition. *P. maculatus* was shown to lay 140 to 230 eggs per spawning and the mean size of fertilized egg was 1.6 mm. The egg requires atleast 48 h for hatching and the hatchling size is 3.9 mm where the yolk absorption complete in three days. After absorption of yolk only the larvae accept external food (Bindu and Padmakumar, 2012). The biology of *P. maculatus* is well suited as laboratory model in toxicology studies as it is highly sensitive to the changing environmental conditions (Nandan and Nimila, 2012; Asifa and Chitra, 2016; Sumi and Chitra, 2017). Unfortunately, overexploitation of the species for commercial purposes and pollution in aquatic ecosystems has threatened its existence.

1.11 Objectives and structure of the thesis

Generally, toxicity tests are used to evaluate the toxic potential of environmental contaminants, including pesticides. The toxicity can be determined by the application of the toxicants on the skin, inhalation, oral administration, or by intravenous or intramuscular injection into the body of experimental organisms. In the case of animals living in water or aquatic medium, the toxicant is directly dissolved in it. Therefore, assessment of toxicity is different from those of terrestrial animals. In order to test the toxicity of chemicals, Organization for Economic Co-operation and Development (OECD) had putforth guidelines for aquatic animals. According to the guidelines, toxicity tests can be performed in several ways as acute, sub-acute and chronic toxicity.

Acute toxicity tests are the initial screening tests for the manifestation of toxic characteristics of a chemical. In this test, single or multiple dose of the

chemical is used in test animal for short-term durations by oral, dermal, inhalation or parenteral exposures. It provides data on the relative toxicity for the determination of gross behaviour and lethality (Bhardwaj and Gupta, 2012). According to OECD guidelines, commonly used acute toxicity measures are median lethal dose (LD_{50}) and median lethal concentration (LC_{50}) for 96 h durations. This value denotes the amount of a toxicant either in the form of lethal dose (LD) or concentration (LC), which kills 50% of the population of the test animal within a fixed period of time (Finney, 1971; Akhila *et al.,* 2007). Median lethal dose (LD_{50}) is the test performed in terrestrial animals by the administration of test chemical generally through oral, inhalation or intramuscular or any other parenteral methods. However, if the test animals are aquatic, the toxicant will be generally mixed with the surrounding medium and the toxicity is expressed in terms of median lethal concentration (LC_{50}). According to OECD test guideline-203 or equivalent (1992), acute aquatic toxicity would normally be determined using fish by 96 hour LC_{50}. LD_{50} is expressed in milligrams or micrograms of product per kilogram of body weight (mg/kg or µg/kg) whereas LC_{50} is expressed in terms of milligrams or micrograms of chemical per litre of water or any other medium (mg/L or µg/L). The lower LD_{50} or LC_{50} value, higher the toxicity of the chemical.

The following conditions are prescribed by OECD guidelines for determining the LC_{50}. They are:

- At the end of the test, the mortality in the control(s) should not exceed 10 per cent.
- Constant conditions should be maintained throughout the test.
- Concentration of the dissolved oxygen must have been at least 60 per cent of the air saturation value throughout the test.
- Concentration of the substance being tested has been satisfactorily maintained throughout the experiment (OECD, 2000).

Other measures of acute toxicity are NOEL, ED_{50} and EC_{50} (Holt *et al.,* 2002). No Observed Effect Level (NOEL) is the exposure level at which there is no statistically or biologically significant increases in the frequency or severity of any effect between the exposed population and its appropriate control. It is an important

measure, in cases where LD_{50} and LC_{50} values do not indicate toxicity of a compound over the range toxicant concentrations (Holt *et al.*, 2002). Median effective dose (ED_{50}) and median effective concentration (EC_{50}) are the amount/concentration of a compound where 50% of the tested population exhibits a toxic response, after specified exposure duration. Thus acute toxicity data are mainly used to identify the lethal or toxic dose of a chemical which in turn provide a rough guide for selecting experimental or sublethal dose for further toxicity studies. Chronic toxicity tests involve continuous contact to low levels of chemical over a long period of time. Chronic effects may be prolonged for over a period of months or even years.

The field of aquatic ecotoxicology has developed over the past several decades. It has gone from simple and naive science in the 1960s and '70s, to a much more erudite and mechanistic discipline with the advent of new technologies and methods. Behavioural toxicology has developed as an auspicious discipline to link the laboratory to field. Generally, animal behaviour incorporates the internal physiology of the animal and the external conditions of the environment. Toxicant prompted behavioural impairments frequently plug to underlying physiological discrepancies which can be used effectively to assess ecological risk (Pyle and Ford, 2017). Thus behavioural modifications are important manifestation of acute toxicity caused by environmental contaminants. In recent years, correlation of behavioral sciences with toxicity studies has become predominant in aquatic ecotoxicology studies (Melvin and Wilson, 2013). Moreover, behavioral modifications are reflected as a receptive sign of stress conditions in animals exposed to toxicants (Beitinger, 1990).

The objectives of the research strictly followed the guidelines approved by OECD. Two sublethal concentrations chosen after the acute toxicity test was used as experimental dose. The objectives of the thesis include the following:

- To evaluate the effects of chlordecone on male reproductive system of fish
- To assess the interference of chlordecone on female reproductive system of fish
- To determine the hormonal influence of chlordecone in fish.

- To study the consequences of chlordecone on the antioxidant status of non-reproductive and reproductive tissues of fish.

The above parameters are discussed in separate chapters as follows:

Chapter 2: Effects of chlordecone on the reproductive system of the fish, *Pseudetroplus maculatus*.

Chapter 3: Hormonal changes induced by chlordecone in *Pseudetroplus maculatus.*

Chapter 4: Effects of chlordecone on antioxidant status in reproductive tissues.

Chapter 5: Effects of chlordecone on antioxidant status in non-reproductive tissues.

Chapter 6: Effects of chlordecone on metabolic enzyme activities.

Chapter 7: Effects of chlordecone on histopathology of reproductive and non-reproductive tissues.

Review of Literature

Over the past few decades aquatic pollution is one of the public health issues faced globally. A wide variety of harmful chemicals are continuously released into water bodies from various sources. In developing countries, 70% industrial wastes are dumped into natural water bodies, without any further treatments which generally pollute the usable water resources to cause severe health problems (Connor *et al.,* 2017). The United Nations reports that economic cost of problems due to water pollution is very severe in the Asia-Pacific region in terms of renovating the quality of life and establishing control measures (UN, 1998). Thus water pollution and reduction of freshwater resources are considered as an important environmental problem in Asian region (ADB, 1997). In India, most of the surface waters including rivers, ponds and lakes are highly polluted owing to anthropogenic activities of different origin. Studies have reported that almost 80% of the water bodies are highly polluted in India. Important rivers such as Ganga, Hoogly, Yamuna, Damodar and Gomati are now toxic dumps of highly polluting industries, waste water treatment plants and municipal corporations which are situated near their banks (Goel, 2006). Thus our aquatic resources become ultimate sink of a wide variety of harmful environmental contaminants.

Most of these chemicals are lipophilic and resistant to degradation so that they can easily enter the organisms through several routes, bioconcentrate and cause deaths and diseases together with severe pathological conditions including cancers and reproductive abnormalities (Chance, 2001; Gray *et al.,* 2001). Pesticides have been known to cause oxidative stress, behavioural modifications, reproductive impairments, endocrine disruption, histopathological alterations along with several biochemical, enzymatic and haematological consequences in fishes and other aquatic organisms (Sinha *et al.,* 1991; Chitra *et al.,* 2001; Das and Mukherjee, 2003; Jiraungkoorskul *et al.,* 2003; Scott and Sloman, 2004; Lal, 2007; Slaninova *et al.,* 2009; Raibeemol and Chitra, 2015; Ramya *et al.,* 2015). Exposure to endocrine disrupting chemicals has been shown to cause a wide variety of species-specific and tissue-specific toxicity such as alterations in gonadal development, impaired reproductive performances, hormonal variations, carcinogenesis, changes in immunity, behaviour and skeletal deformities (Arcand-Hoy and Benson, 1998;

Armour *et al.,* 2001; Scholz and Kluver, 2009; Soto and Sonnenschein, 2010; Clayton *et al.,* 2011; Frye *et al.,* 2012). Several pesticides are known to act as endocrine disruptors and the degree of endocrine disrupting potential may vary based on their chemical nature and physiology of the species exposed.

Application of acute toxicity tests for evaluating the potential threat of such endocrine disruptors in aquatic organisms is well documented. In the case of aquatic organisms median lethal concentration (LC_{50}) is the commonly used acute toxicity test for determining the lethality of contaminants. It describes the relationship between mean mortality and concentration of toxicant at specific acute exposure period (Finney, 1971). Previous reports have suggested that the median lethal dose for rabbit is 71 mg/kg, which is lower than that for rats, dogs or chicks (Larson *et al.*, 1979; Sherman and Ross, 1961). When rats were fed with chlordecone at 50 ppm through diet has showed the death of the animal within six months. However, when mice received chlordecone above 80 ppm through diet died within 32 days (Huber, 1965).

The presence of chlordecone in the environment has been assumed due to the extensive use of mirex, which may contain chlordecone as a contaminant or the photodegradation of mirex to form chlordecone (Carlson *et al.,* 1976). When chlordecone-containing diet ranging from 0.25 to 5.0 ppm fed to cow, the levels of chlordecone in blood began to appear at day 4 and the dose increased to 20 to 320 ng/ml after 60 days (Smith and Arant, 1967). The degree of tremor produced in the animal as well as the inhibition of ATPase activity has been reversed when chlordecone was removed by washing or by the addition of anti-chlordecone antibodies (Koch *et al*., 1977). Exposure to chlordecone has been shown to cause neurological symptoms such as nervousness, headaches, and tremors in humans (Cannon *et al*., 1978). The metabolism of chlordecone to chlordecone alcohol occurs in humans, pigs and gerbils at a greater extend and less significantly in rats, mice, hamsters or guinea pigs (Blanke *et al.,* 1978; Fariss *et al.,* 1980; Houston *et al.,* 1981). In epidemiological studies it was reported that chlordecone in a reduced form was noted in the stool of the workers and they experienced symptoms of toxicity, including headache, nervousness and tremor (Blanke *et al.,* 1978). Intoxication of

chlordecone has been associated with the symptoms of pseudotumour cerebri that includes severe headache, pressure in spinal fluid, which are apparently due to impaired capacity to absorb cerebrospinal fluid (Sanborn *et al*., 1979). Effects of chlordecone in rats also include competitive inhibition of muscle lactate dehydrogenase and the myopathic effects of chlordecone are found to be permanent and time-dependent (Egle *et al*., 1979). Administration of daily doses of chlordecone at 50, 25, or 10 mg/kg body weight showed 90% of mortality within 5, 9, or 24 days, respectively (Huang *et al*., 1980). Chlordecone has been proved as neurotoxic in mouse and it was due to inhibition of synaptosomal membrane Na+, K+ ATPase(s), which would then result in blocked cellular uptake and storage of neurotransmitters as catecholamines or γ-aminobutyric acid (Ho *et al*., 1981). Similarly, the neurotoxicity of chlordecone has been reported in adult and juvenile rat brain by regulating the Na+/K+-ATPase activity (Bansal and Desaiah, 1985).

Chlordecone induced crucial threat for aquatic ecosystems because of its stability and persistence in sediments, bioaccumulation in food chains, and its acute and chronic toxicity. Estrogenicity of chlordecone is proved by several *in vivo* studies in rats, in which chlordecone competitively inhibited the binding of estradiol to uterine cytosolic estrogen receptors suggesting that chlordecone readily binds to the same receptor site as estradiol. Moreover, incubation of chlordecone with uterus *in vitro* has been shown to increase the estrogen receptors in nuclear fraction with concomitant decline in the concentration of estrogen receptors in cytosolic fraction indicating the translocation of estrogen receptors (Bulger *et al*., 1979). Estrogenic activity of chlordecone is found associated with the decrease in the reproductive potential of animals especially males, which is evidenced by the reduced fertility and fecundity (Goodman *et al*.., 1982).

Chlordecone at 15 and 30ppm concentrations in rats showed decreased motility and viability of epididymal spermatozoa and sperm reserves in the cauda epididymidis (Linder *et al.,* 1983). The metabolism of chlordecone to chlordecone alcohol has been demonstrated in pig by the intraperitoneal administration of chlordecone at dose of 40 or 80 mg/kg/day for 35 day durations in the blood and gallbladder bile (Soine *et al.,* 1983). Chlordecone reductase activity was detected in

the liver cytosol of rabbits, gerbils, and humans but was absent in rats, mice, hamsters, and guinea pigs (Molowa *et al.,* 1986b). After entering into the body of organisms, chlordecone has been found to accumulate in hepatic nuclei and increased cytochrome P-450 activity in rats (Crouch and Ebel, 1987). Chlordecone and 17β-estradiol induced increased atresia among vitellogenic follicles in the ovaries of mouse, which could be due to the estrogenic properties of chlordecone (Swartz and Mall, 1989). In contrast with this, one of the earlier studies has reported that chlordecone is anti-estrogenic in rat pituitary (Huang and Nelson, 1986). Chlordecone is a persistent estrogenic pesticide which has the potential to alter the functioning of endocrine system in carp (Smeets *et al*., 1999).

Chlordecone has been found to act as an agonist of G-protein coupled estrogen receptor 1 and up-regulated adenylyl cyclase activity (Thomas and Dong, 2006). Exposure of chlordecone in human mainly occurs through food, especially by consuming aquatic organisms and root vegetables contaminated with the toxicant (Dubuisson *et al*., 2007). Chlordecone alcohol is excreted from the body primarily through biliary route into feces as glucuronide conjugate and as compared with other tissues it has been shown to be eliminated more slowly from the liver in rats (Belfiore *et al.,* 2007). Studies have reported that increased risk of prostate cancer is associated with increase in the concentration of chlordecone in blood plasma (Multigner *et al*., 2010). The freshwater prawn *Macrobrachium rosenbergii* exposed to chlordecone for 8 days caused induction of genes involved in the expression of cytochrome P450 and glutathione-S-transferase (GST) (Gaume *et al.,* 2015).

Chlordecone has been shown to accumulate in prawn tissues and functions similar to the effects of estrogen by affecting growth and reproduction in both male and female *Macrobrachium rosenbergii*, which is further evidenced by the induction in the expression of genes for vitellogenin and vitellogenin receptors along with the reduction in the concentration of 20-hydroxyecdysone (Lafontaine *et al.,* 2016b). The ecotoxicological impact of the organochlorine chlordecone has been also evaluated on soil microbial community that induced deviations in structure, abundance, and function thereby indicating microbial toxicity (Merlin *et al*., 2016). Chlordecone exposure has been shown to cause hepatotoxicity, neurotoxicity,

reproductive impairments and disturbances in early child development in humans and other animals (Guzelian, 1982; Nedellec *et al.,* 2016; Tabet *et al.,* 2016). Endocrine disruption function of chlordecone has been evidenced by showing resemblances to estrogen receptors and cytochrome P450 which in turn caused increase in the concentration of plasma estradiol, induction of vitellogenin in females and degeneration of gonads in both male and female rare minnow (Yang *et al*., 2016).

Exposure of chlordecone in hepatopancreas of *Macrobrachium rosenbergii* revealed that about 6% of proteins involved in important physiological processes such as normal functioning of endocrine system and hormonal control of reproduction or development processes were down-regulated thereby indicating the proteomic response to chlordecone (Lafontaine *et al.,* 2017). One of the recent studies has discovered an important possible remediation for soil contamination of chlordecone by *in situ* chemical reduction process. The study reported that dechlorinated chlordecone derivatives are devoid of genotoxicity and mutagenicity and have lesser proangiogenic properties compared to the parent compound (Legeay *et al.,* 2017). Based on the literatures reviewed, it is unspoken fact that chlordecone is estrogenic endocrine disruptors but the lack of supporting evidence on the acute toxicity effects of chlordecone in the fish, *Pseudetroplus maculatus* is a gap area. Thus the task of the present study is to consistently evaluate the acute toxicity by using simplified methods in order to improve the understanding of toxic effects of chlordecone.

Materials and Methods

3.1 Experimental animal

Adult male and female freshwater cichlid fish, *Pseudetroplus maculatus*, weighing 7±1 g and 7±1.5 cm in length were collected from Kondan Kulavan Fishfarm Nursery, Manjeri, Vaniyambalam, Kerala, India. Fishes were brought to the laboratory with least disturbance and were acclimatized to the laboratory conditions (12 h light: 12 h dark) for 15 days prior to experiments. They were maintained in dechlorinated, well-aerated aquarium tanks (40 L capacity) and the health status of the animal was continuously monitored throughout the experiment.

3.2 Preliminary tests

Preliminary tests were conducted by maintaining water temperature at 28±2°C, oxygen saturation of water (70 and 100 %), and pH 6.5 to 7.5 using standardized procedures as per American Public Health Association (APHA, 1998) guidelines. The standardized physico-chemical features were maintained throughout the experiment in both control and treated groups.

3.3 Chemicals

Technical grade organochloride pesticide, chlordecone (Kepone, decachlorooctahydro-1,3,4-metheno-2H-cyclobuta [cd]-pentalen-2-one, 99.9% purity) was obtained from Supelco, USA. All other chemicals were of analytical grade and obtained from local commercial sources.

3.4 Evaluation of median lethal concentration (LC_{50}-96 h)

The concentration of any toxicant that is lethal to one-half of the test population is referred to as median lethal concentration (LC_{50}) or median tolerance limit. In the experiment, the concentration of chlordecone at which 50 percentage of the exposed fish undergo mortality at a specific period, i.e., for 96 h is LC_{50}-96 h or median lethal concentration of chlordecone. Fishes were not fed a day prior to and during the test to reduce fecal and excess food contaminating the test solution. For determining LC_{50} concentration, separate tanks of tap water (40 L capacity) were taken, which was dechlorinated and aerated using tubed motorized pumps.

Monofilament netting was used to cover the tanks so as to prevent the specimens from jumping out of test solutions. Six different concentrations (25, 30, 35, 40, 50 and 80 µg/ L) of chlordecone were added in each separate tank. Chlordecone was dissolved in 1% DMSO (dimethyl sulfoxide) as chlordecone is moderately soluble in water. Thus, DMSO was used as the solvent or vehicle (positive) control in the experiment. Control tank, without toxicant, and vehicle control (DMSO) were also maintained along with the treatment groups. In both controls and experimental tanks, 10 fishes were introduced and mortality as well as the behaviour of fishes was recorded throughout the study. The lethal concentration for 50% killing (LC_{50}) values was computed on the basis of probit analysis for 96 h with a confident limit of 5 % level (Finney, 1971).

The above acute toxicity tests were repeated three times in order to confirm the mortality and to reduce the statistical errors. Data were analyzed by Probit of regression analysis as statistical method using Statistical Package for the Social Sciences, SPSS 19.0 statistical analysis software. The LC_{50} value (with 95% confidence limits) was calculated, and then the correlation between mortality against concentrations and the best-fit line were also obtained.

3.5 Selection of test concentrations and durations

The median lethal concentration of chlordecone for 96 h (LC_{50}-96 h) in *Pseudetroplus maculatus* determined by probit analysis was 35 µg/ L (Asifa and Chitra, 2015a). Based on the LC_{50}-96 h value, two sublethal concentrations such as, one-tenth (3.5 µg/ L) and one-fifth (7 µg/ L) of LC_{50} concentration were selected. Fishes were exposed to chlordecone at both sublethal concentrations for short-term and long-term durations. Short-term exposure includes 24, 72 and 96 h durations and long-term exposure includes 4, 7, 15 and 30 days. Analysis of sperm parameters in males, activities of steroidogenic enzymes in ovary and testis, metabolic enzyme activities, assay of antioxidant enzymes and histopathology of reproductive and non-reproductive tissues were evaluated in short-term treatment. Hormonal parameters, concentration of vitellogenin in plasma of male and female fishes were performed after long-term exposure.

3.6 Experimental design

Parameters	Exposure period (hours/ days)	Test concentrations (Chlordecone µg/L)	Number of samples
• Sperm parameters in males • Activities of steroidogenic enzymes in ovary and testis • Assay of antioxidant enzymes in reproductive and non-reproductive tissues • Metabolic enzyme activities • Histopathology of reproductive and non-reproductive tissues	Short-term (24, 72 and 96 h)	Controls One-tenth of LC_{50}-96 h (3.5 µg/ L) One-fifth of LC_{50}-96 h (7 µg/ L)	N = 10/ group
• Concentration of vitellogenin in plasma of male and female fishes • Hormonal parameters	Long-term (4, 7, 15 and 30 days)	Controls One-tenth of LC_{50}-96 h (3.5 µg/ L) One-fifth of LC_{50}-96 h (7 µg/ L)	N = 10/ group

3.7 Sample collection

At the end of every treatment period, fishes were caught very gently using a small dip net, one at a time with least disturbance. Body weights were recorded immediately after the end of every exposure period. Mucous deposition over the surface of the body was noted. The weights of organs such as gill, liver and brain in non-reproductive tissues and the weights of reproductive tissues namely, ovary and testis were documented in control groups and treatment groups. Respective tissues were processed according to the requirement of the parameters, which was detailed in the respective chapters. Blood collected by cardiac puncture and sperm samples

collected from urinogenital tract of controls and treatment groups were used for respective analysis as explained in the separate chapters.

3.8 Statistical analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control groups. Data are presented as mean ± SD for ten animals per group. All experiments were carried out in duplicates to minimize statistical errors.

Results

4.1 Effect of chlordecone on acute toxicity in the fish, *Pseudetroplus maculatus*

Different concentrations of chlordecone showed different percentage of mortality at different time interval as shown in Table 1. The body weight of the fish remained unchanged throughout the experiment maintaining the range of 7±1 g. Mortality of the fishes in each group were continuously monitored throughout the experiment and it was observed that at 25 µg/ L concentration of chlordecone showed no mortality at 96 h whereas at 50 and 80 µg/ L showed 100% mortality of the animal after 40h and 8h of chlordecone exposure. Chlordecone at concentrations 30, 35 and 40 µg/ L showed death of 3, 5 and 7 animals respectively at the end of 96h exposure. From this, it was clear that 50% mortality was obtained at 35 µg/ L concentration of chlordecone. The results were further confirmed by probit analysis which showed LC_{50} value of chlordecone as 35 µg/ L. The values are also plotted as graph and the results of correlation analysis showed that mortality (X-variable) against concentrations of chlordecone (Y-variable) was highly positive correlation, where the r value is +0.8468 (Figure 1). Thus it was observed that at the concentrations above 50 µg/ L showed 100% mortality.

4.2 Effect of chlordecone on behaviour of the fish, *Pseudetroplus maculatus*

The behaviour of the fishes in chlordecone treated tank showed a drastic alteration when compared to the control groups. All the fishes showed spontaneous swimming activity at the beginning of the treatment and it gradually decreased to become lethargic. All the fishes interact with each other and knocked on the walls of the tank frequently, and finally showed erratic swimming at the time of death. Reddened eyes and fins, especially caudal fin, and exophthalmic eyes were observed after chlordecone exposure (Figure 2). Hemorrhage on the body surface, high degree of mucus production, increased surfacing i.e., restricted movement of the fish at the surface of water, decreased rate of opercular movement, inability to maintain normal posture and balance are the behavioral changes that was observed during chlordecone treatment.

Table 1 **Percentage of fish mortality exposed at different concentrations of chlordecone for 96 h**

Concentrations (µg/ L)	Mortality (No. of animals)	Mortality (%)	Hour of mortality	Probability
Control	0	0	96 h	0
DMSO	0	0	96 h	0
25	0	0	96 h	0.03622
30	3	30	96 h	0.19580
35	5	50	96 h	0.47518
40	7	70	48 h	0.73432
50	10	100	40 h	0.96213
80	10	100	8 h	0.99999

Figure 1: **Median lethal concentration of chlordecone for 96 h in the fish, *Pseudetroplus maculatus***

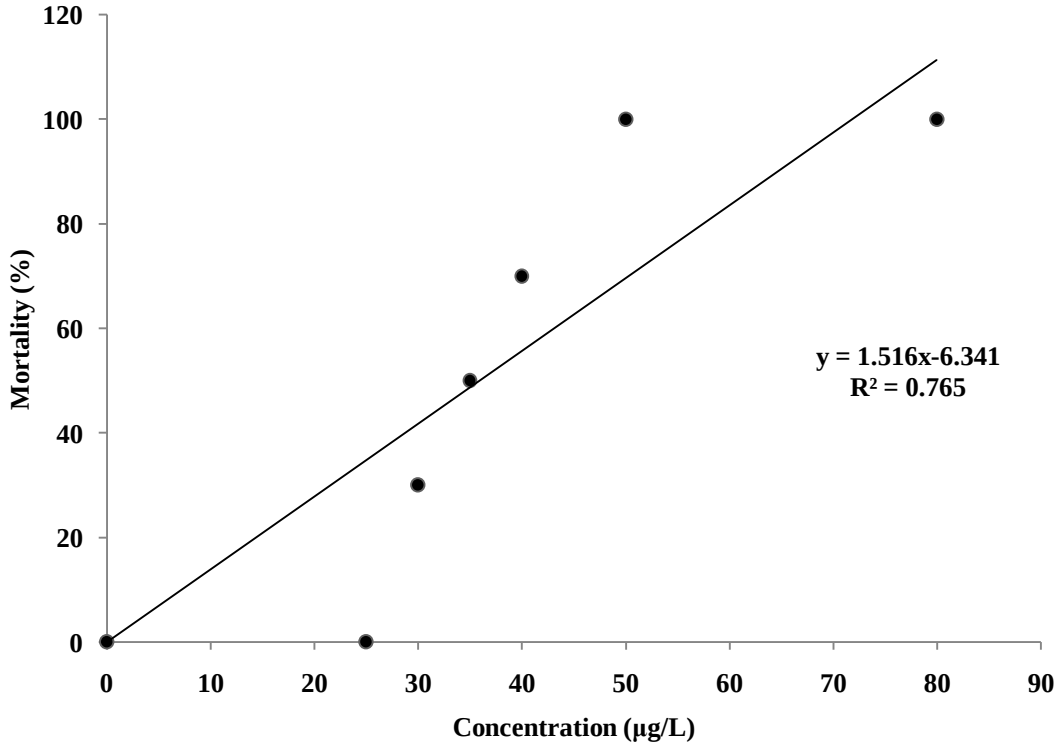
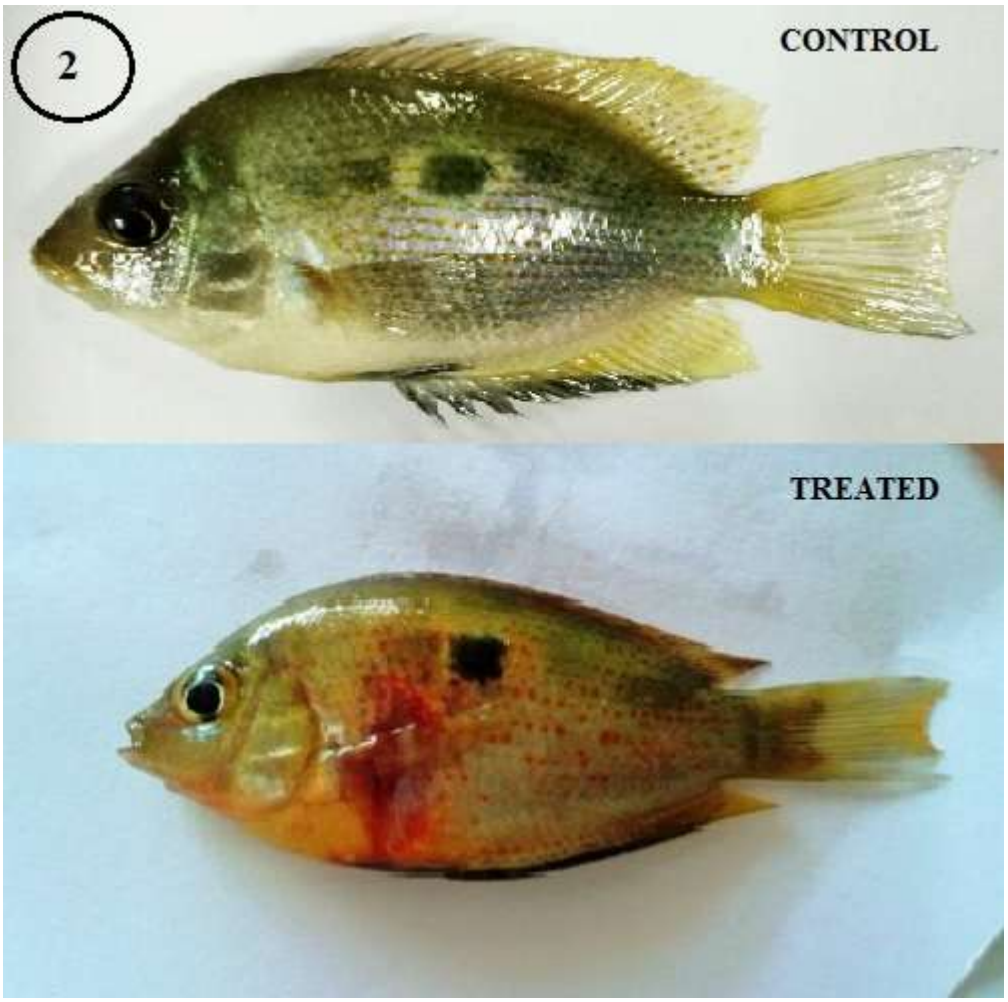


Figure 2: Exophthalmic and hemorrhagic body of chlordecone exposed fish



Discussion

5.1 Acute toxicity of chlordecone in the fish, *Pseudetroplus maculatus*

According to OECD guidelines, for testing acute toxicity of any test chemicals, fish are exposed to toxicants preferably for 96 h and the mortality of fish are recorded for 96 h at 24 h interval. The percentage at which the test chemical kills 50% of the fish in a group is determined as median lethal dose or median lethal concentration of the test chemical (OECD, 1992). In ecotoxicology studies, the acute test has been a major component in toxicity testing for a period of 96 h. Acute toxicity test of contaminants have been regularly performed on fish in order to obtain quick estimates of the concentrations that lead to direct, irreversible damage to the tested organisms (Pandey *et al.*, 2005). Fish are considered dead if there is no visible gill movement and if no reaction on touching the caudal peduncle. Dead fish are immediately removed when observed and mortalities are recorded. Observations are done initially at three and six hours after the start of the experiment and then after 24 h interval for 96 h.

In the present study, the median lethal concentration i.e., 96h-LC_{50} value of chlordecone in *Pseudetroplus maculatus* was found to be 35µg/L (Asifa and Chitra, 2015a). LC_{50} of chlordecone was already reported in several other species and it has been shown to vary from species to species. The 96h LC_{50} value of chlordecone in fathead minnow and larval sea lamprey, *Petromyzon marinus*, were reported as 340µg/L (Buckler *et al.*, 1981) and 444µg/L, respectively (Mallatt and Barron, 1988). Similarly, 96h LC_{50} value of chlordecone in *Ictalurus punctatus* (channel catfish), *Lepomis macrochirus* (bluegills), and *Anguilla rostrata* (American eel) were reported as 514µg/L, 50µg/L and 35µg/L, respectively (Roberts and Bendl, 1982). For estuarine copepod *Eurytemora affinis* 48h-LC_{50} was evaluated as 131.61µg/L (Legrand *et al.*, 2017) and for Indian catfish, *Heteropneustes fossilis* 96h-LC_{50} is 0.24mg/L (Srivastava and Srivastava, 1987).

5.2 Behavioural modifications of chlordecone in the fish, *Pseudetroplus maculatus*

Change in the behavioural pattern is the adjustment of organisms to external and internal stimuli in order to meet the challenges of surviving in a changing

environment. In aquatic organisms, behavioral responses have been frequently used as an important endpoint in aquatic toxicology studies and environmental monitoring (Diamond *et al.,* 1990; Gerhardt, 2007). Behaviour of an organism offers a perspective link to the physiology and ecology of an organism and its environment (Sloman and McNeil, 2012). In the present study, chlordecone exposure irrespective of varying concentrations showed abnormal behaviour in fishes when compared to control groups. Immediately after the toxicant exposure fishes showed spontaneous swimming for a few hours, which is considered as the instant behavioural response to escape or avoid the area containing the toxicant. But, the fishes are unable to avoid the toxicant area as the animal was maintained in tanks, which would resulted in slow and restricted swimming activity, increased surfacing with decreased rate of opercular movement thereby altering the respiratory pattern. These behavioural changes appear to be an effort by the fish to avoid breathing in the toxicant contaminated water, which in turn reduce the absorption of poison through the gills leading to reduced rate of oxygen consumption (Singh *et al*., 2009).

Organophosphate pesticides such as chlorpyrifos induced abnormal swimming activity, which is triggered by the lack of muscular and nervous co-ordination that may be due to the accumulation of acetylcholine in synaptic and muscular junctions (Rao *et al*., 2005). Increased secretion of mucous is considered as the first line defensive mechanism to prevent the entry of chlordecone into the body either through gill, or skin. Mucous cells are considered efficient in seizing the toxic agents and thus help to prevent the entry of these toxicants into the gills (Perry and Laurent, 1993). After 96h of chlordecone exposure body surfaces and fins become reddened and hemorrhagic and the animal fails to maintain normal posture or equilibrium. Then the fishes experience jerky and hyperexcitable movement before collapsing, settle at the bottom of the tank with loss of consciousness, lethargic and finally resulted in mortality. These changes are due to the adverse toxic effects of chlordecone on different vital organs. Similar observations such as restricted movements, haemorrhagic on entire body surface, reddening of fins and finally loss of equilibrium have been reported when nonylphenol and bisphenol A

was exposed to the fish, *Etroplus maculatus* (Asifa and Chitra, 2015b; Asifa *et al*., 2016c).

Short-term exposure of herbicides like atrazine and diuron has been shown to alter the swimming activity along with decreased grouping behavior and increased surfacing activity in goldfish (Saglio and Trijasse, 1998). Similarly, *Labeo rohita* when exposed to a biopesticide azadiracht exhibited erratic swimming activity, decreased rate of opercular movement, increased surfacing, reduced agility and inability to maintain normal posture and balance with increasing exposure time (Bhat *et al.*, 2012). Thus behavioural responses are important indicator of acute toxicity caused by various contaminants in aquatic ecosystem.

To brief, acute toxicity testing is the statistical endpoint to determine the toxicity of chlordecone in the fish, *Pseudetroplus maculatus*, which when determined by probit analysis was 35 µg/L. The mortality rate of the fish was found to be increased with the increase in concentration of the toxicant along with the behavioural modifications.

Conclusions

1. The cichlid fish, *Pseudetrolpus maculatus* is highly sensitive to the organochlorine pesticide chlordecone.

2. The acute toxicity of median lethal concentration (LC_{50}) for 96 h of chlordecone is determined as 35µg/ L by probit analysis.

3. It is the lowest median lethal concentration value among other aquatic species, which reveals the high toxicity of chlordecone in the fish.

4. The behavioural manifestations caused by chlordecone intoxication in fish are considered as an important indicator of contaminant induced toxicity.

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CHAPTER 2

Effects of chlordecone on the reproductive system of the fish

Introduction

1.1 Description of male reproductive system

Inter-specific and intra-specific variations in gonads of fish depend on several features like change in morphology or anatomy of gonads as well as modification in the environmental conditions in which they live (Rahemo and Al-Shatter, 2012). The structure of testis in teleost fishes differs among the species and is classified as lobular and tubular testis, based on the differentiation of germinal tissue (Grier, 1981). In lower teleosts, the germinal compartment is organized in the form of tubules whereas in higher groups it is arranged as branching lobules (Grier, 1993). In most of the fish, testes are paired, elongated and sac-like structures which are located in the midline of the body. Based on the reproductive cycles, the size and morphology of testis differs, which comprises different phases such as early maturation, mid maturation, late maturation, and regression phase (Uribe *et al*., 2014). Generally, testis consists of germinal compartment and interstitial compartment, which are separated by a basement membrane. The germinal compartment or lobule is formed by germinal epithelium which contains germ cells and somatic epithelial cells (Grier and Uribe, 2009). Therefore, the inner part of testicular lobule is made up of germ cells in the form of cysts which is surrounded by somatic epithelial cells. The process of spermatogenesis occurs within the germ cells, which contain spermatogonia and all other stages of differentiation of the spermatogenic cells that finally transform into spermatozoa (Schulz *et al*., 2010).

The somatic epithelial cells are called Sertoli cells which offer nutrients to the developing germ cells and provide permeability barrier throughout the process of spermatogenesis (Grier, 1993). Sertoli cells also support in the development, survival and physiological functioning of germ cells, phagocytosis of apoptotic germ cells and residual bodies in the seminiferous tubule and also involved in the secretion of certain fluids that generate the tubular lumen (Schulz *et al*., 2010). The ultrastructure of Sertoli cells of fish reveals the presence of spherical mitochondria with parallel cristae and lipid deposits in the cytoplasm which are the fundamental features of steroid-producing cells, advocating a potential role in steroid biosynthesis or sites where the steroid hormones are stored (Da Cruz-Hofling and Da Cruz-Landim, 1984).

The interstitial compartment contains connective tissue and Leydig cells. The connective tissue encloses fibroblasts, collagen fibres, immunological cells like macrophages and lymphocytes, myoid cells, blood vessels and nerve fibres. The Leydig cells are positioned particularly close to the blood vessels which are involved in the process of steroidogenesis leading to the production of testosterone, which is the key hormone responsible for normal development and functioning of testis (Van Den Hurk, 1973). Most of the fishes do not possess seminiferous tubules, which are the structural unit of testis in higher vertebrates. Instead, fishes possess certain spherical structures called sperm ampullae in which the sperm are produced. Sperm ampullae are functional and develop only during breeding season and after performing the function, it is reabsorbed by the body. The new sperm ampullae then begin to develop and ripen before the next breeding season. The presence of similar kind of cells in the sperm ampullae made it identical to that of seminiferous tubules of higher vertebrates (Romer and Parsons, 1977). The testis then continues as a narrow duct namely sperm duct or vas deferens which later opens outside by urethra. In some fishes, mesonephric ducts fuse with testis to form vas deferens and vasa efferentia or epididymis. While some groups of teleost fishes possess Wolffian duct which is formed by sharing the sperm duct with kidney and often called nephric duct (Wourms, 1977). The two important functions performed by testis are spermatogenesis and steroidogenesis, which are very crucial for the perpetuation of life.

1.2 Spermatogenesis

Spermatogenesis is the complex process that occurs in the seminiferous tubule of testis where the production of haploid spermatozoa from diploid spermatogonia occurs by repeated mitotic division followed by meiotic division. Most of the anamniote vertebrates, including fishes, the mode of spermatogenesis occur by cystic type, in which the process of spermatogenesis progress inside a cyst of Sertoli cells (Callard, 1996). Thus the fundamental functional unit of the spermatogenic epithelium in fish is the spermatogenic cyst formed by an active group of Sertoli cells, which encircles a group of germ cells containing a single

syncytium that synchronously divides and differentiates. The histology of fish testis shows different developmental stages of spermatogenesis having dissimilar sized germ cells in tubular compartments (Schulz *et al.,* 2010). The spermatogonia within the tubule undergo repeated mitotic divisions to produce cysts, so that each cyst contains several generations of spermatogonia such as undifferentiated, differentiated and differentiating spermatogonia. Only the fully matured spermatogonia undergo meiotic division to produce primary and secondary spermatocytes, which is followed by the final differentiation process called spermiogenesis. During the final differentiation of spermatids several morphological, physiological and biochemical changes occur in spermatids to become mature spermatozoa. It involves development of flagellum, condensation of DNA into a small nucleus, deposition of extracellular contents into residual body, enlargement of cysts and finally rupturing of cyst for the release of spermatozoa into the lobular lumen continuous with the sperm duct (Nagahama *et al*., 1994).

Most of the tropical fishes exhibit obvious seasonal variations in spermatogenic activity. In many species with different habitats, reproduction is either seasonal or cyclic event that are correlated with environmental conditions (Billard and Breton, 1978). *Pseudetroplus maculatus*, the experimental model used in the present study is reported to reproduce throughout the year within the backwaters of Kerala (Jayaprakas *et al*., 1979). The process of spermatogenesis is controlled by the co-ordinated action of several endocrine glands and the hormones produced by testis. Therefore, the process of spermatogenesis is under the control of male hormone, testosterone, produced by the Leydig cells of testis.

1.3 Testicular steroidogenesis

Steroidogenesis is the process by which cholesterol is converted to biologically active steroid hormones with the involvement of several enzymes. In fish, biosynthesis of sex steroid hormones mainly take place in specialized cells of testes namely Leydig cells, granulosa and theca cells of ovarian follicles and zona reticularis of adrenal tissue (Young *et al.,* 2005). Cholesterol is the fundamental precursor for the biosynthesis of all steroid hormones. Steroidogenesis is initiated by

the import of cholesterol from outer to the inner mitochondrial membrane, which is facilitated by steroidogenic acute regulatory (StAR) protein (Lin *et al*., 1995). Generally, StAR protein acts on the outer mitochondrial membrane to bind with cholesterol and after transportation into the mitochondria, it gets inactivated (Bose *et al*., 2002). Within the inner mitochondrial membrane, cholesterol is converted to the basal steroid, pregnenolone by side chain cleavage enzyme, cytochrome P450scc which is a major enzyme of monooxygenase family (Parker and Schimmer, 1995). Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme found in both mitochondria and smooth endoplasmic reticulum. Pregnenolone and progesterone constitutes the precursors for the other steroid hormones where they undergo a series of biochemical reactions mediated by various cytochrome P450 enzymes and dehydrogenases in the Leydig cells to form testosterone (Dharia *et al.,* 2004). The weak androgen androstenedione, an intermediate product of steroidogenic pathway is finally converted to testosterone by 17β- hydroxysteroid dehydrogenase (17β-HSD) enzyme (Mindnich *et al*., 2004). Therefore, both 3β-HSD and 17β-HSD plays an important role in testicular steroidogenesis.

1.4 Fish spermatozoa

Like other vertebrates, fish spermatozoa consist of head, middle piece and flagellum. The head region contains DNA and midpiece contains mitochondria together with the proximal and distal centrioles. Flagellum originates from the basal body and consists of 9+2 pairs of the microtubules (Lahnsteiner and Patzner, 2008). In most of the fish species with external fertilization, the spermatozoa are quiescent and immotile in the seminal plasma (Stoss, 1983). It becomes motile and metabolically active only when it is released into the surrounding aqueous environment during natural reproduction or into the diluents during artificial reproduction (Alavi and Cosson, 2006). Induction of motility is influenced by several external environment factors such as osmolality, concentration of specific ions, pH, temperature and cations, which varies from species to species (Morisawa and Suzuki, 1980; Morita *et al*., 2003; Alavi *et al*., 2008; Le *et al.*, 2011). All these

environmental factors trigger motility in spermatozoa by depolarizing the cell membrane. Usually, the duration of motility in fish is very short and it has been reported to last for less than two minutes following activation (Alavi and Cosson, 2005).

The period of motility is very crucial in fishes because male fertility primarily depends on sperm motility parameters (Billard *et al*., 1995). Similar to motility, the other important indices essential for the success of reproduction includes viability and concentration of spermatozoa. Non-viable or immotile sperm are incapable to undergo fertilization and embryo production. Sperm concentration, which is nothing but, the number of spermatozoa per milt volume, varies from species to species (Lubzens *et al*., 1997; Hulak *et al*., 2008) and it could be due to the variation in the production of seminal plasma (Shaliutina *et al.,* 2012). Other parameters, such as DNA integrity and level of oxidative damage are also important for the fertilizing potential of spermatozoa (Shaliutina, 2013). Since, spermatozoa parameters are highly sensitive to the change in the environmental conditions including chemical contaminations, studying these parameters could offer valuable information to evaluate toxicity of xenobiotics on male reproduction of fish.

1.5 Description of female reproductive system

The female reproductive system of fish composed of a pair of ovaries which contain a hollow, sac-like, lymph-filled space which opens into the oviduct. The oviduct opens outside by urinogenital aperture through which the eggs are released (Romer and Parsons, 1977). At the anterior end, the ovaries are free whereas they are fused to form the oviduct at posterior end, which opens outside through the urinogenital aperture. Projecting into the lumen of ovaries, there are numerous ovigerous lamellae, which are formed by the folding of germinal epithelium. Ovigerous lamellae possess numerous oogonia and oocytes at different stages of differentiation and maturation (Tokarz, 1978). The main two types of ovaries found in teleost include gymnovarian and cystovarian type. In gymnovarian type, the ovulated eggs are shed directly into the abdominal cavity and released outside through the genital pore (Kagawa, 2013). Cystovarian type is the most predominant

type of ovary found in teleost fishes, where the lumen of ovary continues with the oviduct through which the oocytes are released outside (Brito and Bazzoli, 2003; Guimaraes-Cruz *et al*., 2005).

The ovary consists of two forms of cells namely somatic cells and germ line cells. The main components of somatic cells include ovarian capsule and interstitial cells. The ovarian follicles developed from the germinal epithelium contain diploid oogonia, which are derived from primordial germ cells (Nishimura and Tanaka, 2014). The diploid oogonia undergo several mitotic divisions followed by meiotic division to form the oocytes. As in other vertebrates, the teleost oocytes are enveloped by two layers of follicle cells namely an outer thecal layer and an inner granulosa cells. Generally, the ovary contains numerous oocytes in different stages of development by the process called oogenesis (Hoar *et al*., 1983).

1.6 Oogenesis

Primordial germ cells in the ovary undergo several structural and molecular changes for the formation of mature and functional ovum. It is initiated by the transformation of primordial germ cells to diploid oogonia by mitosis and each oogonium multiplies again mitotically to form the oogonial nest in connection with pre-granulosa cells. Following the last mitotic division, the diploid oogonium proceeds first meiotic division to form the primary oocyte within the oogonial nest (Guraya, 1986). Finally, the young oocyte separates from the oogonial nest and bounded by a single layer of somatic granulosa cells, which secrete a basement lamina to set apart from the ovarian stroma cells. Outside the basal lamina, the somatic cells also form a monolayer cells called theca, which are associated with the blood vessels. Thus, the oocyte enveloped by granulosa cells, basement lamina and theca constitutes the ovarian follicle (Le Menn *et al.,* 2007). Granulosa cells are specialized somatic cells covering primary oocytes, which along with thecal cells play a key role in ovarian steroidogenesis (Nagahama *et al*., 1995). In this stage, primary oocytes are arrested at the diplotene stage of first meiotic division for few days to months depending on the species and start the next stage of growth and maturation (Babin *et al*., 2007). During the growth period, the oocytes undergo

several structural and molecular changes along with the accumulation of nutritional reserves needed for the development of the embryo through a process called vitellogenesis.

1.7 Growth of oocyte

The growth of ovarian follicle is divided into primary and secondary growth phases, which are also called previtellogenic and vitellogenic stages, respectively. The primary growth stage of oocyte is characterized by the increase in the size of nucleus with multiple alveoli termed as perinucleolar stage. In this stage, large amounts of rRNA and mRNAs are produced, which encode proteins for the further growth of oocytes, formation of lampbrush chromosomes, accumulation of basophilic and electron-dense material in the perinuclear cytoplasm, increased oocyte volume with decreased nucleo-cytoplasmic ratio and elaborated membranous organelles in the cytoplasm (Wallace and Selman, 1981). The increase in cell size is due to the formation of Balbiani bodies, which are cytoplasmic masses corresponding to various cellular organelles like endoplasmic reticulum, Golgi apparatus, cisternae, multivesicular bodies and lipid granules (Fard *et al.*, 2013). Lipid deposition that is derived from the very low density lipoprotein in circulation also begins at the time of pre-vitellogenic phase. At the end of pre-vitellogenic stage, the oocytes begin to accumulate neutral lipids, which are stored as lipid droplets in the ooplasm (Reading and Sullivan, 2011). The secondary growth phase starts with the appearance of certain vesicles called cortical alveoli which fills the periphery of the oocyte (Selman *et al.,* 1993). These are membrane-bound vesicles of variable size and contain glycoprotein synthesized by the oocyte itself. During the cortical reaction of fertilization, the contents of the cortical alveoli are released to the egg surface, which leads to the restructuring of egg envelope proteins forming the chorion (Selman *et al*., 1993). At the time of oocyte growth and maturation, vitellogenin, a major protein that is synthesized in liver is transported to developing oocyte by one of the major events in oocyte called vitellogenesis. The synthesis of vitellogenin by liver and the process of vitellogenesis are under the control of hormones.

1.8 Vitellogenesis

Vitellogenesis is the process of sequestration and packaging of hepatically-derived protein precursor, vitellogenin (Vtg), into yolk protein within the ooplasm, which ensures the embryonic development of fertilized eggs (Wallace, 1978). It is a seasonal or cyclic phenomenon and its duration extends from months to years that are extremely variable among the species. Vitellogenin is a phospholipoglycoprotein of about 300-600 kDa, synthesized by the liver and transported through the blood stream into the ovary, from which it is taken up by oocytes. Vitellogenins enter growing oocytes by selective endocytosis which is mediated by specific membrane receptors in the oocyte membrane called vitellogenin receptors (VtgRs). Within the ooplasm, it is processed into egg yolk proteins under the control of endocrine system (Babin *et al.,* 2007). The yolk proteins are generally stored in the yolk granules, platelets, globules, or fluid yolk of the ooplasm and represent the major protein constituent of the egg yolk. At the end of vitellogenesis, the ovary is filled with yolky oocytes, which later undergo maturation and ovulation under favourable environmental conditions. At this stage, the follicular layers as well as cells of granulosa and theca are well developed and clearly visible.

In order to detect the level of vitellogenin, several techniques are developed globally, which include Enzyme-linked immunosorbent assay (Nilsen *et al*., 2004), immunoassays (Bon *et al*., 1997; Brion *et al*., 2000), immune agglutination (Le Bail and Breton, 1981) and densitometry following electrophoresis (Van Bohemen *et al*., 1981; Allner *et al.,* 1999). Owing to the complexity of vitellogenin molecule, requirement of species-specific antibodies and unaffordable costs are some of the limitations of the assay to restrict in certain species. Thus as an alternative, indirect endpoints such as plasma protein, alkali-labile phosphoprotein, plasma calcium and gonadosomatic index were frequently used to study the induction of vitellogenin in fishes (Verslycke *et al*., 2002). Generally little or no vitellogenin is detected in the plasma of male and immature female fish; however, male fish carry the gene for vitellogenin. The gene also gets activated when the fish is exposed to the environmental estrogenic compounds. Gonadosomatic index provide an additional indicator for the evaluation of endocrine disruptors in the reproduction of fish.

1.9 Maturation of oocyte

After the completion of growth, oocyte undergoes several maturational processes prior to ovulation. At this stage, the oocytes attain maximum size, lose its spherical shape and become flattened. Maturation phase is characterized by reduced or termination of endocytosis, resumption of meiosis, breakdown of germinal vesicle, formation of a monolayer cortical alveoli in the periphery of ooplasm, increase of oocyte volume and dissolution of yolk platelets (Nagahama and Yamashita, 2008). During this stage, the first meiotic division resumes and the primary oocyte divide into two cells with different size. The large cell is called secondary oocyte and the small one called first polar body which degenerate later (Fard *et al.*, 2013). The secondary oocyte undergoes second meiotic division and further undergoes maturation to form haploid ovum and second polar body, which also degenerate soon. Finally, ovulation occurs at the end of maturation processes by collapsing the follicular layers and the ovum is expelled into the ovarian lumen or abdominal cavity depending on the species and finally released outside through urino-genital aperture. The maturation of ovum is arrested in second meiotic metaphase, which is capable of fertilization and usually the binding of sperm facilitates completion of meiosis (McMillan, 2007).

1.10 Ovarian steroidogenesis

Similar to testicular steroidogenesis, cholesterol is the basic precursor for the ovarian steroidogenesis. It occurs in the interstitial cells, granulosa cells and thecal cells in different species of fishes (Yaron, 1971; Lambert *et al.*, 1978; Nagahama and Yamashita, 2008). These cells produce large amounts of progesterone and androgens, which act as precursor for the biosynthesis of potent female hormone, estrogen. The combined action of aromatase, 3β-HSD and 17β-HSD converts androstenedione and testosterone into estradiol within the granulosa cells **(**Kishida and Callard, 2001; Luu-The, 2001). Cytochrome P450-11A (CYP11A) and aromatase (CYP19a and CYP19b) are considered as rate limiting enzymes essential for the production of various steroid hormones and 17β-estradiol, respectively. CYP17α-hydroxylase/ 17, 20-lyase (CYP17) has been shown to regulate the shift

between androgen and estrogen biosynthesis, which is very essential for the early germ cell development to the final germ cell maturation (Miller, 1988). Therefore, 3β-HSD and 17β-HSD plays an important role in regulating ovarian steroidogenesis.

Taken together, in the present chapter the following parameters were analyzed and discussed:

- ✓ Estrogenic effects of chlordecone was assessed by measuring the concentration of vitellogenin in the blood plasma of *P. maculatus* by using simple and reliable biochemical procedures for 4, 7, 15 and 30 days treatment.
- ✓ The present study determines the effects of chlordecone at two sublethal concentrations for 24, 72 and 96 h on the activities of key steroidogenic enzymes, 3β-HSD and 17β-HSD in the testis and ovary of *Pseudetroplus maculatus*.
- ✓ The acute effects of chlordecone on various sperm parameters such as sperm motility, viability and concentrations were evaluated at both sublethal concentrations for 24, 72 and 96 h.

Review of Literature

The major sources of estrogenic environmental contaminants include agricultural runoff, municipal sewage effluents and industrial wastes that are released directly to the aquatic ecosystems. Several studies have reported that the presence of low levels of estrogenic compounds in the aquatic environments have been associated with abnormalities in the reproductive system of fish. Thus reproduction in fish represents one of the sensitive tools to assess the toxicity of various anthropogenic pollutants possessing estrogenic properties in aquatic organisms (Kime, 1995). Some important biomarkers which are widely used to assess the reproductive toxicity of estrogenic contaminants include evaluation of morphology of gonads, vitellogenesis, gametogenesis, spermatozoa parameters like motility, viability, concentration and DNA integrity, number of eggs and viability, oocyte maturation, ovulation, spawning and gestation, and also the normal balance of various hormones of hypothalamo-pituitary gonadal axis and reproductive behaviour (Kime, 1995). Both laboratory and field studies have been conducted in different species of fishes in order to assess the reproductive impairments caused by different pollutants.

The endpoints of reproductive toxicity as a result of the exposure to the pesticide chlordecone have been reported in several species of animals. Chlordecone exposure has been shown to upset sperm production and motility in employees of chlordecone manufacturing factory at Hopewell and similar effects were found in rats after experimental exposure (Cohn *et al.,* 1978; Linder *et al*., 1983). Chlordecone exposure at 0.78mg/L caused reduced fecundity and fertility of eggs as well as decreased survival of embryos in sheepshead minnow, *Cyprinodon variegatus* (Goodman *et al*., 1982). One of the mercuric fungicide, Emisan® when exposed to *Channa punctatus,* showed abnormal development of gonads, which is evidenced by degenerative changes in the histology of pituitary gonadotrophs and oocytes along with reduced gonadosomatic index (Ram and Sathyanesan, 1986). Endosulfan, one of the most toxic pesticides, has been shown to cause alterations in sexual behaviour and oocyte development in *Colisa fasciatus* (Pandey, 1988). Some environmental contaminants are endocrine disruptors, which impair fertility and reproduction in wide variety of animals. These chemicals have the potential to

disturb sensitive hormone pathways in animals, which in turn could affect the reproductive processes. Polychlorinated biphenyl has been shown to decrease the secretion of pituitary gonadotropin and estradiol while cadmium exposure increased the levels of hormones in female Atlantic croaker, *Micropogonias undulates*, accompanied by retarded ovarian growth and decline in vitellogenin synthesis (Thomas, 1989).

In United Kingdom, increased incidence of intersexuality has been reported in wild populations of riverine fish, *Rutilus rutilus*. Most of the male fishes within the observed population are reported as intersex, having both male and female gonadal characteristics together with increased vitellogenin concentration suggesting contamination of rivers with estrogenic constituents of sewage effluents (Jobling *et al.*, 1998). In general, the major target of endocrine disrupting chemicals in fish has been identified as hypothalamo-pituitary-gonadal axis and also in liver to some extent (Pait and Nelson, 2002). Exposure to methylmercury in fathead minnows decreased the serum concentrations of testosterone in male and estradiol in female thereby leading to suppressed gonadal development, decreased gonadosomatic index and reproductive success (Drevnick and Sandheinrich, 2003). Zebrafish when exposed to different concentrations of bisphenol AF has been shown to decrease the concentration of testosterone and increased the concentrations of triiodothyronin and estradiol in males. In females, the toxicant exposure has been shown to increase the titre of both testosterone and estradiol in concentration-dependent manner (Yang *et al.*, 2016). Similarly, one of the carbamate insecticides, methomyl, increased the serum level of estradiol and decreased testosterone and 11-ketotestosterone concentrations in male tilapia (Meng *et al.*, 2017).

The estrogenic contaminants have been shown to alter the secretion and activity of steroid hormones mainly by interfering with the process of steroidogenesis in both male and female fishes. Major steroidogenic enzymes include cytochrome P450 enzymes (CYPs), hydroxysteroid dehydrogenases (HSDs), and steroid reductases (Miller, 1988). Major HSDs such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) play an important role in reproduction in vertebrates, especially in the biosynthesis of sex

steroids. 3β-HSD is also known as Δ5-4-isomerase, which catalyzes the oxidative conversion of Δ5-3β-hydroxysteroids, for example, pregnenolone, 17a-hydroxypregnenolone and dehydroepiandrosterone to the Δ4-3-ketosteroides such as progesterone, 17α-hydroxyprogesterone and, androstenedione. Thus it is essential for the biosynthesis of all classes of hormonal steroids, namely progesterone, glucocorticoids, mineralocorticoids, androgens, and estrogens (Lachance *et al.*, 1992; Norris, 2007). 17β-HSD expressed in the Leydig cells of testes has been known to catalyze the conversion of weak androgen, androstenedione to testosterone (Mindnich *et al*., 2004). Within the thecal cells of ovary, androstenedione and testosterone diffuse into the neighboring granulosa cells where they are converted to estradiol with the help of aromatase and 17β-HSD. Apart from this, 17β-HSD also catalyze the conversion of estrone to estradiol (Luu-The, 2001; Mindnich *et al*., 2004). Several environmental contaminants such as tributyltin, polychlorinated biphenyls and mercury are known to inhibit the activity of 3β-HSD (Mondal *et al.*, 1997; Andric *et al*., 2000; McVey and Cooke, 2003). Also, studies have reported that plant flavonoids including daidzein, biochanin A, formononetin and genistein inhibited the activities of the steroidogenic enzymes *in vitro* (Wong and Keung, 1999; Ohno *et al.*, 2002; 2004). One of the estrogenic environmental contaminants, methoxychlor has been shown to inhibit the activities of both 3β-HSD and 17β-HSD in humans and rats (Hu *et al*., 2011; Latchoumycandane *et al*., 2002). Therefore, steroidogenic enzymes are critical for the biosynthesis of sex steroids and any change in the activities might serve as a sensitive indicator of reproductive dysfunction.

Another extensively documented effect of estrogenic endocrine disruptors includes abnormal vitellogenin production in male fish and hence considered as an important biomarker of estrogenic contaminants (Sumpter and Jobling, 1995). Vitellogenins are dimeric protein, which consists of two identical subunits possessing several components such as phosphate, lipid, carbohydrate and protein. It is an egg yolk protein precursor present in oviparous organisms, including fishes. In female fish, the plasma concentrations of vitellogenin increase progressively during sexual maturation with concomitant increase in estradiol concentrations (Scott and

Sumpter, 1983). The female fish require such a high level of vitellogenins for proper growth of ovary with thousands of yolky oocytes whereas, the concentration of vitellogenin is very less or absent in males. However, exposure to environmental estrogens has been shown to activate vitellogenin gene in males, and triggers for the excessive production of vitellogenin. For example, exposure to 17β-ethinylestradiol at 0.1 ng/L concentration has been reported to cause 500 to 100,000-fold increase in the production of vitellogenin in trouts (Purdom *et al.,* 1994). Exposure of male rainbow trout, *Oncorhynchus mykiss* to alkylphenolic chemicals caused synthesis of vitellogenin in males along with concomitant inhibition of testicular growth (Jobling *et al.,* 1996). Male *Cyprinus carpio* captured near to sewage treatment plant in Minnesota had elevated concentration of plasma vitellogenin and decreased testosterone level compared to fish in unpolluted site (Folmar *et al.,* 1996). Usually, estradiol stimulates production of vitellogenin in females, whereas in males xenoestrogen acts as the source of estradiol directly or may alter the process of sex steroidogenesis for inducing vitellogenesis. Thus abnormal levels of vitellogenin in fishes affects the quality and quantity of gametes leading to reproductive failure and also affect the sexual differentiation in exposed larvae thereby leading to infertility (Kime and Nash, 1999). Bisphenol A when exposed for long-term at concentrations 640 and 1280 μg/L in fathead minnows, *Pimephales promelas,* has been shown to cause the induction of vitellogenin and reduction in spermatogenesis in males with inhibition of gonadal growth in both sexes, while females showed reduced egg production (Sohoni *et al.,* 2001). Male zebrafish, *Danio rerio,* when exposed to high concentrations of diethylhexyl phthalate at 5000 mg/kg body weight for 10 days showed increased expression of the gene for vitellogenin (Uren-Webster *et al.*, 2010).

In males, spermatozoa indices constitute one of the principal targets of environmental contaminants in fishes. Sperm quality is defined as the characteristics of sperm which govern its capacity to fertilize eggs and is critical to predict male reproductive success, which change naturally over breeding season. Usually, motility of spermatozoa is considered as the major factor responsible for the fertilizing ability of sperm (Cosson *et al.,* 1991). Any damage in morphology of

sperm may lead to motility dysfunction, which immensely influences male fertility (Linhart *et al*., 2005). Fish sperm have limited ability to withstand the physico-chemical changes in their external environment, which make it highly vulnerable to disturbances caused by the environmental contaminants (Heath, 1995). There is increasing evidence that several contaminants are known to alter the quality of sperm in fish leading to irreversible reproductive impairments. Many literatures demonstrated that there is significant association between exposure to environmental chemicals and decline in sperm parameters such as sperm count, motility, and viability (Kime *et al*., 1996; Bayley *et al.*, 2002; Rurangwa *et al*., 2002). Spermatozoa anomalies such as incomplete chromatin condensation, changes in head morphology, distorted axonemal structure, head-to-head and head-to-tail sperm agglutinations, wavy and broken plasma membrane and deformed middle piece has been observed in fishes caught from Lake Mariut, Alexandria, Egypt, contaminated with heavy metals copper, zinc and mercury (Abdelmeguid *et al.*, 2007).

The timing of contaminant exposure is also very critical factor in reproductive toxicity because it strongly influences at different stages of reproduction and development in animals. Usually, the reproductive system begins to form during early gestation, but structural and functional maturation is not completed until puberty. The developmental stages, either embryo or larvae, is generally considered as the most sensitive stage in the life cycle of a teleost fishes so that toxicant exposure during this period can lead to alterations that may influence reproductive physiology and functions. A large number of developmental alterations have been reported in fishes during prenatal or postnatal exposure. Examples include exposure to polychlorinated naphthalene in the eggs of medaka has resulted in developmental alterations as decreased gonadosomatic index in early adult life stages (Villalobos *et al.,* 2000). Similarly, development of ova-testis i.e., inter sex was reported in male medaka after maternal exposure to dichloro diphenyl trichloroethane (DDT) (Metcalfe *et al.,* 2000). Studies have found that exposure of *Danio rerio* during 2-60 days after hatching to environmental estrogens, nonylphenol and estradiol, did not affect the sex ratio but occurrence of undeveloped gonads and ova-testes were reported in adults. Exposure to xenoestrogens during

early life stage of zebrafish reported to cause a significant reduction in the number of viable sperm and also decreased rate of hatching in adult fishes suggesting the late consequences of the contaminant (Hill and Janz, 2003). Exposure of pharmaceutical ethynylestradiol at environmentally relevant concentrations to breeding populations of zebrafish showed reduced fecundity and reduced fertility in the F_1 generation due to impaired sexual differentiation with undifferentiated or intersex gonads (Nash *et al.,* 2004). Similarly, developmental exposure to estradiol have been shown to cause reduced fecundity, induction of vitellogenin and formation of intersex in adult Java medaka (Imai *et al.,* 2005)

Contaminant induced structural and functional impairments in the gonads and gametes finally lead to alterations in sexual behaviour, fertilization capacity, spawning, and gestation in fish along with distorted sex ratio and infertility in its offspring (Arukwe, 2001; Sanderson, 2006). Fathead minnows exposed to environmental concentrations of 17α-ethinylestradiol showed altered spawning behaviour along with alterations in sex steroids and vitellogenin synthesis in males (Salierno and Kane, 2009). Chlordecone has been shown to cross placenta which was detected in the cord blood of pregnant women of French West Indies populations resulting in neurological problems in infants (Boucher *et al.,* 2013). Chlordecone treatment at 0.01, 0.1, 1 and 10 μg/L concentrations induced the formation of intersex by the induction of vitellogenin along with decreased gonadosomatic index, degenerated ovaries and increased estradiol/testosterone ratio in both male and female rare minnow, *Gobiocypris rarus* by agonistic interaction with estrogen receptors and cytochrome P450 (CYP19A) (Yang *et al.,* 2016). Prenatal and perinatal exposures to chlordecone has been reported to cause gestational weight gain and alter infant growth which is evidenced by higher body mass index in children aged 0-18 months of Guadeloupe populations (Costet *et al.,* 2015; Herve *et al.,* 2016). The concerns for estrogenic pollutants on reproductive toxicity have been addressed in several literatures; however, the consequence of chlordecone on reproductive physiology in the fish, *Pseudetroplus maculatus* remains scanty and was evaluated in the present chapter.

Materials and Methods

3.1 Chemicals

Bovine serum albumin, trichloroacetic acid, ortho-phosphate were obtained from Sigma-Aldrich, USA. Lanthanum chloride, dehydroisoandrosterone, 1,4-androstenedine-3,17-dione, NAD, NADPH were purchased from Himedia Laboratories, Mumbai, India. Other chemicals were procured from local commercial sources.

3.2 Collection of samples for analysis

Fish were exposed to chlordecone at two sublethal concentrations, 3.5 µg/ L (one-tenth) and 7 µg/ L (one-fifth) for short-term (24, 72 and 96 h) and long-term (4, 7, 15 and 30 days) durations, maintaining negative and positive controls. At the end of every experimental period, fish were killed by decapitation. Body weights of the animal along with mucous all over the body were recorded first, followed by weighing the animal without mucous after clearing the mucous with tissue paper. Mucous deposition was calculated by taking deviations of body weight with and without mucous and expressed in percentage.

Blood collected from both the control and the treatment groups of long-term durations were used for the measurement of vitellogenin concentration. Gonads (testis/ ovary) were dissected out from both short-term and long-term durations, and the weights recorded to calculate gonadosomatic index.

Gonads collected from the short-term durations were cleaned from mucous and debris and homogenates (1% w/ v) of gonads were prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 800 *g* for 15 min at 4ºC so as to obtain the supernatant, which was then used for the determination of steroidogenic enzymes. Sperm samples collected from short-term durations were used to test sperm parameters. The detailed procedures are given below:

3.3 Collection of blood

Blood samples were collected from both male and female fishes in separate clean micro centrifuge tubes using fine syringe by cardiac puncture method. Blood was centrifuged at 1700 *g* for 10 min at 4ºC and plasma samples were collected and stored at ˉ80°C in micro centrifuge tubes for the measurement of vitellogenin concentration.

3.3.1 Analysis of vitellogenin

The total protein concentration in plasma was determined according to the method of Lowry *et al*. (1951) with bovine serum albumin as standard. Alkali-labile phosphoprotein was measured by using the modified method of Wallace and Jared (1968). Briefly, sample (10 µL) was incubated along with trichloroacetic acid (10%) overnight and precipitate obtained was centrifuged at 200 g for 10 min. The precipitate was then incubated again in trichloroacetic acid (5%) for 30 min at 90ºC and washed successfully with hot alcohol, chloroform-ether-alcohol (1:2:2), acetone and ether. The precipitate obtained was dissolved and incubated in sodium hydroxide (2N) at 100ºC and neutralized with an equivalent amount of hydrochloric acid. Absorbance of the solution was measured at 660 nm in spectrophotometer using ortho-phosphate as a standard.

Total plasma calcium levels were measured by atomic absorption spectrophotometry (Spectra-AA240FS, Varian) by the method as described by Zettner and Seligson, 1964. Briefly, volume of the plasma was diluted for 40-fold with 0.1% lanthanum chloride, mixed and centrifuged at 1000 *g* for 10 min and the supernatant was used to estimate calcium levels, and different concentrations of calcium were used as standards. Measurements of calcium content in standards and unknown samples were measured at 422.7 nm in lamp current 3.5mA using acetylene-nitrous oxide as fuel. The standards and samples were aspirated into the burner atomizer through a polyethylene tube connected to the atomizer capillary and three independent readings were taken of each solution on the percentage of absorption counter. The usual sequence was as follows: First, the diluent alone was aspirated and the null meter adjusted so that the

null meter reading was “0” when the percentage of absorption counter read 00.00. Then the percentage of absorption was determined for all standards and samples. The percentage of absorption readings were averaged, converted to absorbance, and the values for calcium concentration were determined with the aid of computer and the values expressed in μg/mL.

3.4 Gonadosomatic index (GSI)

The relative weight of gonads from the control and treated fishes were calculated as follows:

GSI (%) = (Total gonad weight (mg) /total body weight (mg)) × 100

3.5 Activities of steroidogenic enzymes

3.5.1 3β-hydroxysteroid dehydrogenase (3β-HSD)

The activity of 3β-HSD in the gonads was determined by the method as described by Bergmeyer (1974). The reaction mixture contained pyrophosphate buffer (100 mM), NAD (0.5 mM), dehydroisoandrosterone (0.1 mM). The absorbance was read at 340 nm immediately after the addition of enzyme extract at 20 second intervals for 5 min in a spectrophotometer against the blank. Activity of enzyme was expressed as nmol of NAD reduced/ min/ mg protein.

3.5.2 17β-hydroxysteroid dehydrogenase (17β-HSD)

The activity of 17β-HSD was assayed by the method of Bergmeyer (1974). The reaction mixture contained pyrophosphate buffer (100 mM), NADPH (0.5 mM) and 1,4-androstenedine-3,17-dione (0.8 mM). The absorbance was read at 340 nm immediately after the addition of enzyme extract at 30 second intervals for 5 min in a spectrophotometer against the blank. Activity of enzyme was expressed as nmol of NADP formed/ min/ mg protein.

3.6 Collection of sperm

Sperm samples were collected from urinogenital tract by inserting catheter into culture container to prevent contamination. Collected sperm were transferred to a small Petri dish and immediately used for testing the following sperm parameters:

3.6.1 Sperm concentration

Prior to determination of spermatozoa concentration, sperm was diluted 100 times with an immobilizing solution, Tris-Sucrose solution (10: 100 mM at pH 8.5). A droplet (10 µL) of diluted sperm suspension was placed on a counting chamber of the hemocytometer (depth 0.1 mm) with a coverslip. Sperm were counted by using a microscope at 40X magnification and sperm concentration was calculated according to method as described by Caille *et al*. (2006). Spermatozoa concentration was expressed as 10^9 spermatozoa /mL.

3.6.2 Sperm viability

Sperm suspension (20µL) was mixed with an equal volume of 0.05% eosin-Y. After a minute of incubation at room temperature, slides were observed under light microscope at 100X magnification for live and dead sperm and morphological abnormalities were also noted. Viable sperm remained colourless while non-viable sperm stained in eosin appeared pink (Wyrobek *et al*., 1983). Sperm were counted in both control and treatment groups and viability was expressed in percentage (Eliasson, 1977).

3.6.3 Sperm motility

Collected sperm (1 µL) was diluted with distilled water (49 µL) and placed on a Neubauer-type haemocytometer prepositioned on the microscope stage at 40X magnification. Sperm swimming activity was recorded for two minutes, and counted for motile and non-motile sperm. First non-motile sperm was counted followed by motile

sperm and the motility was expressed as a percentage of motile sperm of the total sperm counted.

3.7 Statistical analysis

Statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control groups. Data are presented as mean ± SD for ten animals per group. All experiments were carried out in duplicates to minimize errors.

Results

4.1 Effect of chlordecone on body weights and mucous deposition

Body weight of the animal after exposure to chlordecone decreased significantly ($P<0.05$) at the end of 72 and 96 h after short-term exposure at both sublethal concentrations when compared to the corresponding control groups (Figure 1). Chlordecone when exposed for long-term durations showed significant ($P<0.05$) reduction in the body weights in both time-dependent and concentration-dependent manner when compared with the control groups (Figure 1).

Mucous deposition was found to increase in time-dependent manner when animal was treated with chlordecone, however, significant ($P<0.05$) increase was observed only after 96 h of one-fifth of LC_{50} concentration (Figure 2). Chlordecone exposure up to 30 days showed a time-dependent and concentration-dependent significant ($P<0.05$) increase in mucous deposition, which is 30 – 60% increase when compared to the control groups (Figure 2).

4.2 Effect of chlordecone on vitellogenin concentration

Chlordecone exposure caused significant ($P<0.05$) increase in the levels of plasma calcium, alkali-labile phosphoprotein (ALP) and plasma protein in both males and females in time-dependent and concentration-dependent manner (Figures 3-5). Relationship between different plasma parameters were examined by linear-regression analysis and it showed positive correlation between plasma calcium and alkaline-labile phosphoprotein ($y=0.699x-42.67$; $r=+0.9306$), calcium and plasma protein ($y=0.106x+3.891$; $r=+0.9075$), alkali-labile phosphoprotein and plasma protein ($y=0.143x+10.79$; $r=+0.9181$) in male fishes (Figures 6-8). In female fishes, all plasma parameters were positively correlated showing correlation between plasma calcium and alkali-labile phosphoprotein ($y=0.892x+15.76$; $r=+0.9437$), calcium and plasma protein ($y=0.152x-5.544$; $r=+0.9334$), alkali-labile phosphoprotein and plasma protein ($y=0.152x-5.316$; $r=+0.8833$) (Figures 9-11).

4.3 Effect of chlordecone on gonadosomatic index

Chlordecone exposure at 3.5 μg/ L concentration for 24, 72 and 96 h did not show changes in the gonadosomatic index in both male and female gonads.

However, at one-fifth of LC_{50} concentration showed significant ($P<0.05$) decrease in the gonadosomatic index after 72 and 96 h of chlordecone exposure (Figure 12).

Long-term exposure of chlordecone at both one-tenth and one-fifth of LC_{50}-96 h concentrations showed significant ($P<0.05$) reduction in the gonadosomatic index in testis and ovary of the fish, *Pseudetroplus maculatus* in all treatment groups when compared to the control groups (Figure 13). No remarkable alteration in the gonadosomatic index was observed in the positive control (DMSO-treated) groups in all treatment groups and found similar to that of the negative control (without solvent) group (Figures 12 and 13).

4.4 Effect of chlordecone on the activities of steroidogenic enzymes

The activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase after chlordecone exposure for 24, 72 and 96 h showed significant ($P<0.05$) reduction at both sublethal concentrations in the testis and ovary of the fish (Figures 14-17).

4.5 Effect of chlordecone on sperm parameters

Chlordecone exposure at both sublethal concentrations showed significant ($P<0.05$) decrease in sperm concentration, viability and motility in duration and concentration-dependant manner when compared to the corresponding control groups (Figures 18-20). Sperm when stained with eosin showed colourless viable sperm in control groups and when the exposure period increased the dead sperm appeared pink colour along with morphological abnormalities such as enlarged sperm head and reduced or absence of sperm tail (Figures 21 and 22).

Figure 1: Effect of chlordecone on the body weight of the fish, *Pseudetroplus maculatus*

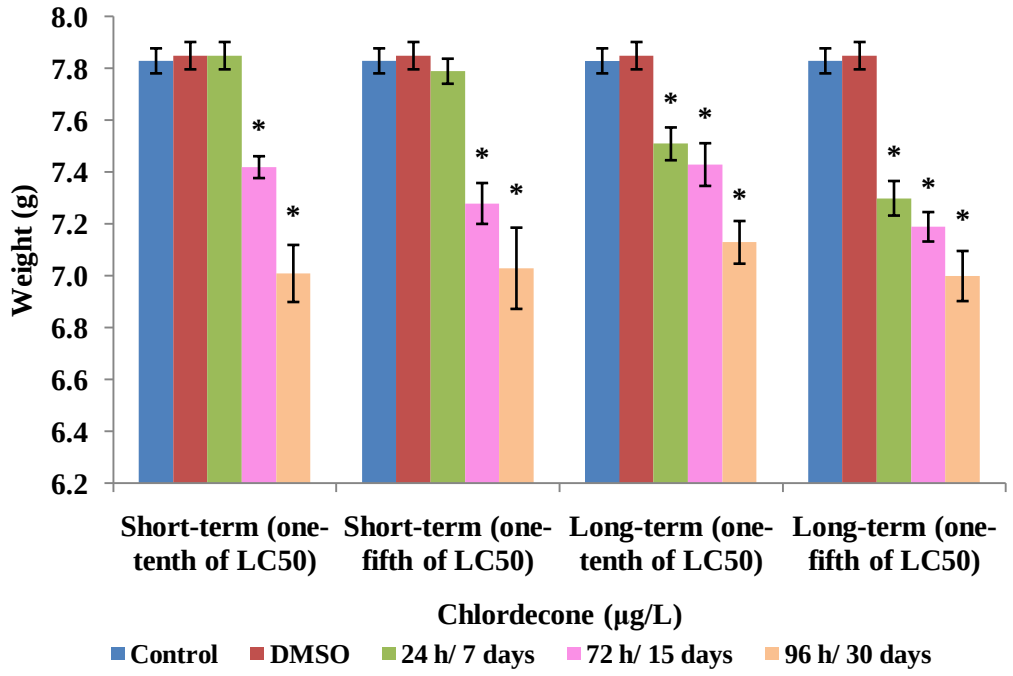


Figure 2: Effect of chlordecone on the mucous deposition in the fish, *Pseudetroplus maculatus*

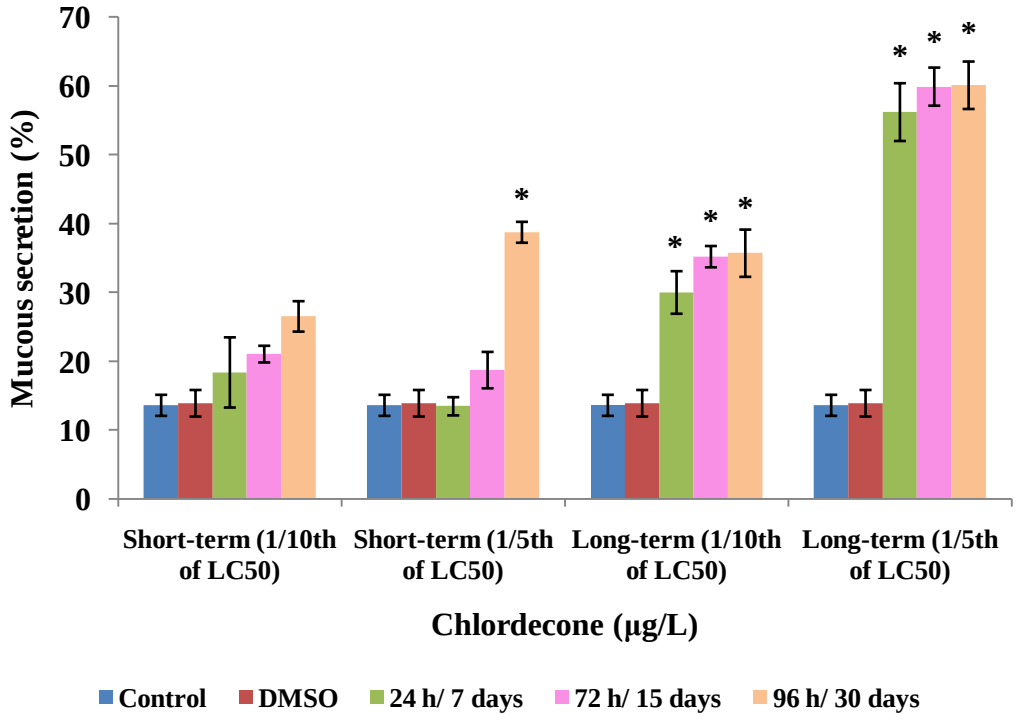


Figure 3: Effect of chlordecone on the level of calcium in the fish, *Pseudetroplus maculatus*

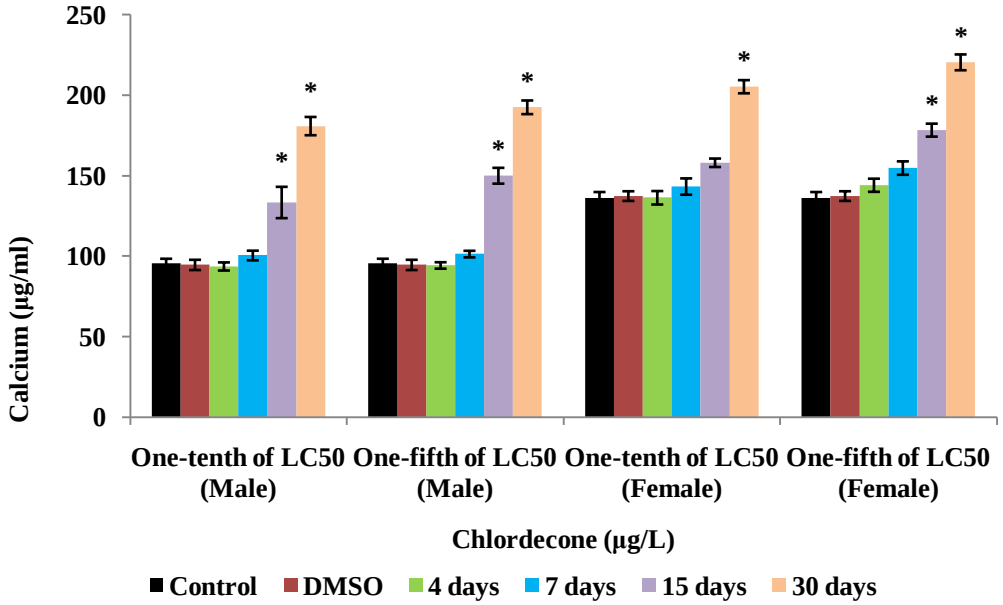


Figure 4: Effect of chlordecone on the level of alkali-labile phosphoprotein in the fish, *Pseudetroplus maculatus*

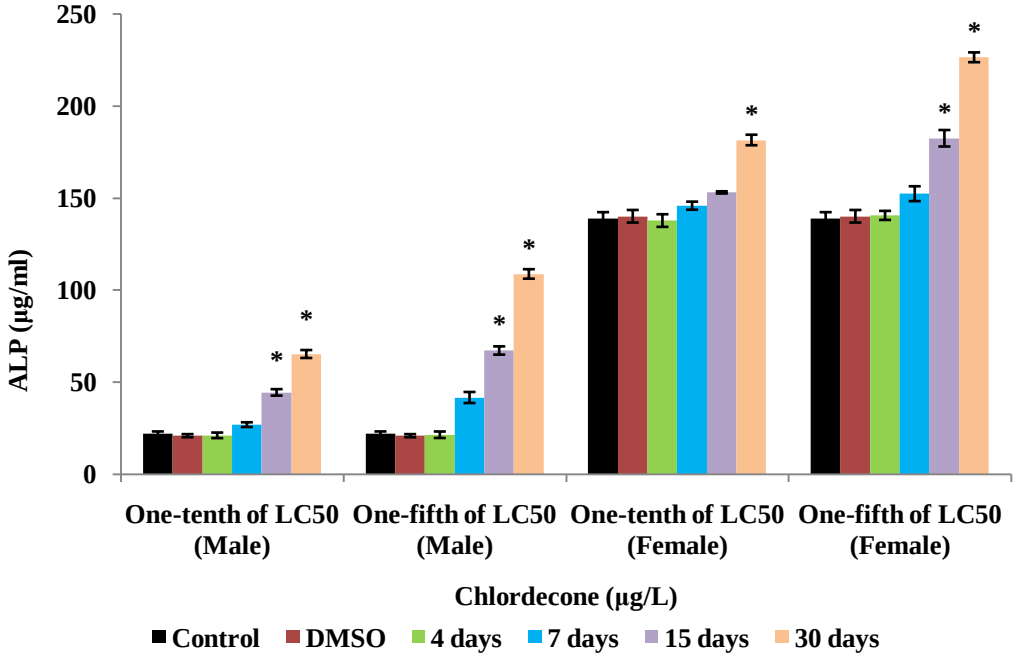


Figure 5: **Effect of chlordecone on the level of plasma protein in the fish, *Pseudetroplus maculatus***

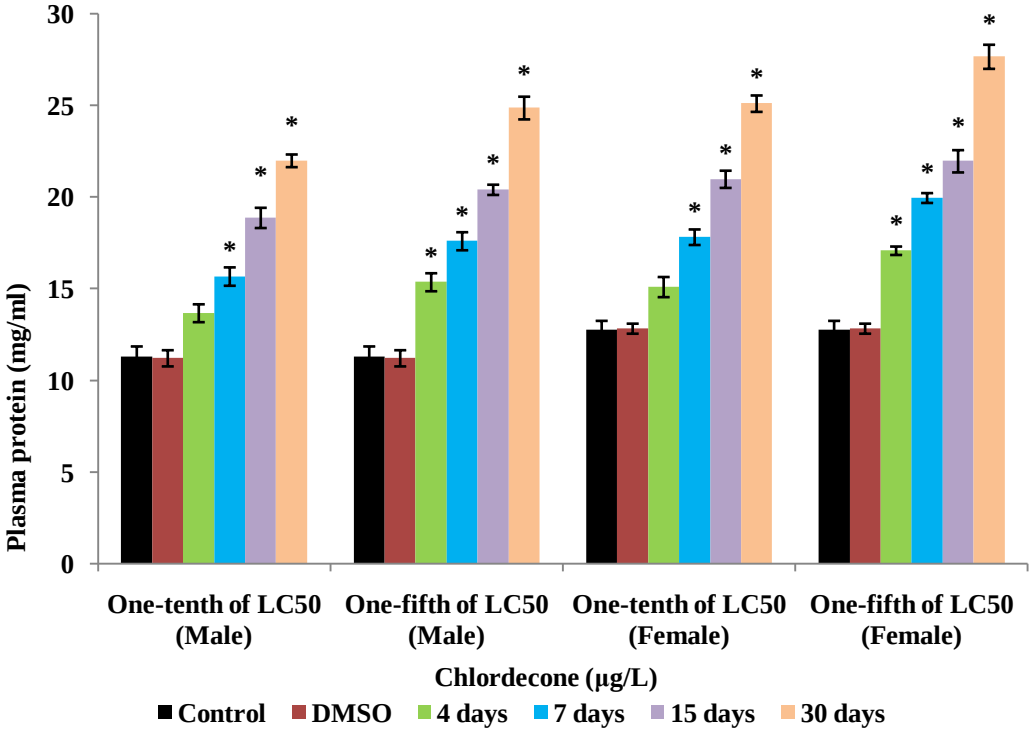


Figure 6: **Correlation between plasma calcium and alkali-labile phosphoprotein exposed to chlordecone in male fish, *Pseudetroplus maculatus***

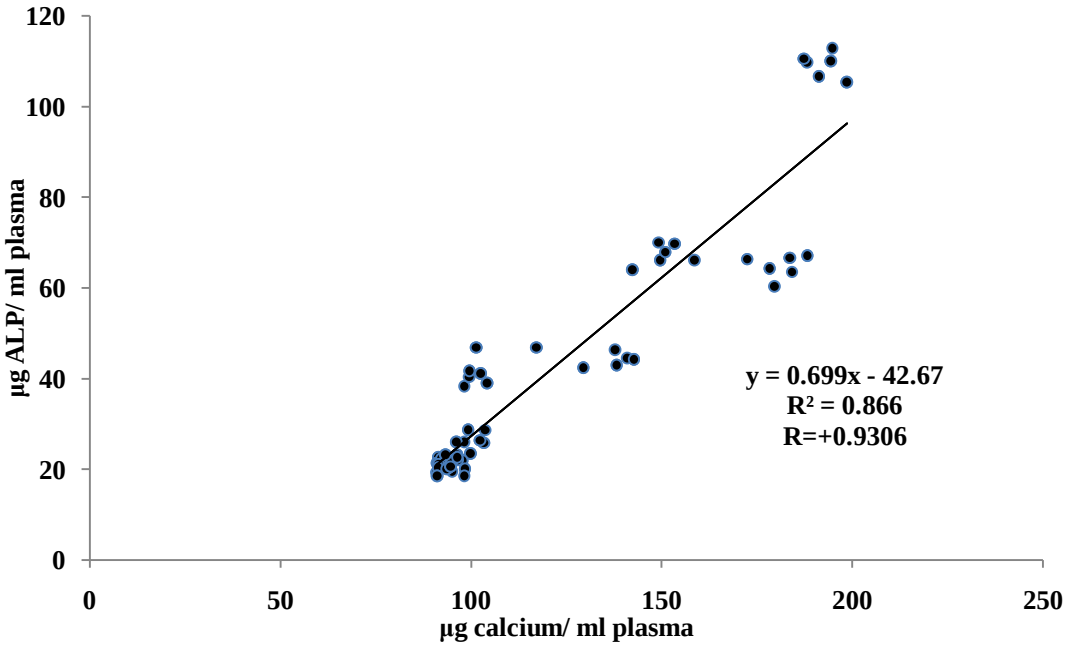


Figure 7: Correlation between calcium and plasma protein exposed to chlordecone in the male fish, *Pseudetroplus maculatus*

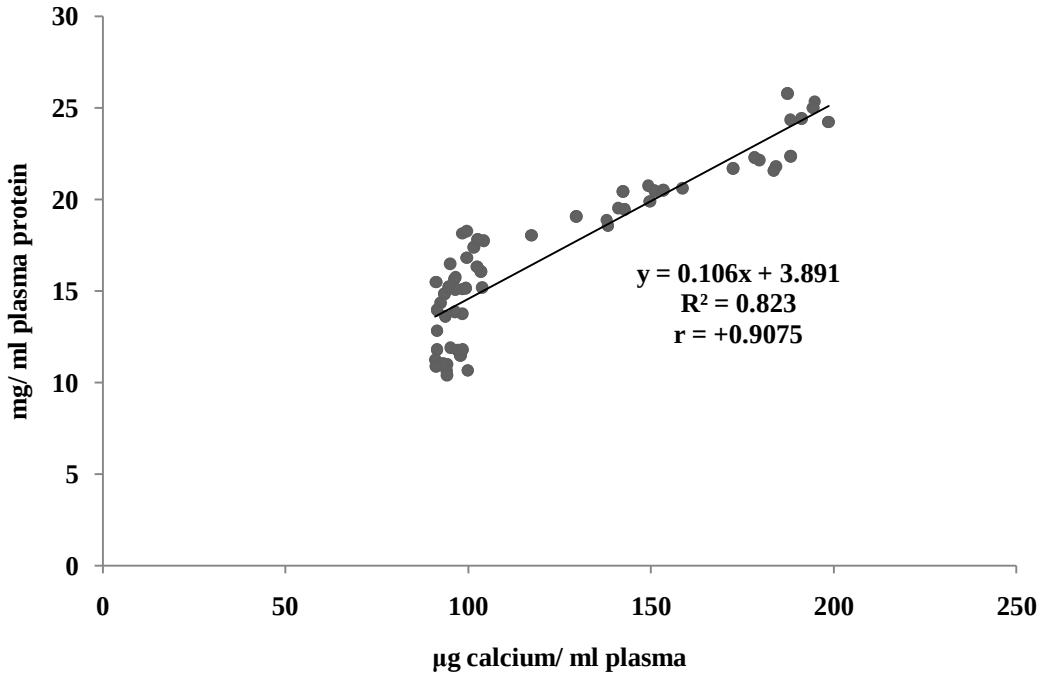


Figure 8: Correlation between alkali-labile phosphoprotein and plasma protein exposed to chlordecone in the male fish, *Pseudetroplus maculatus*

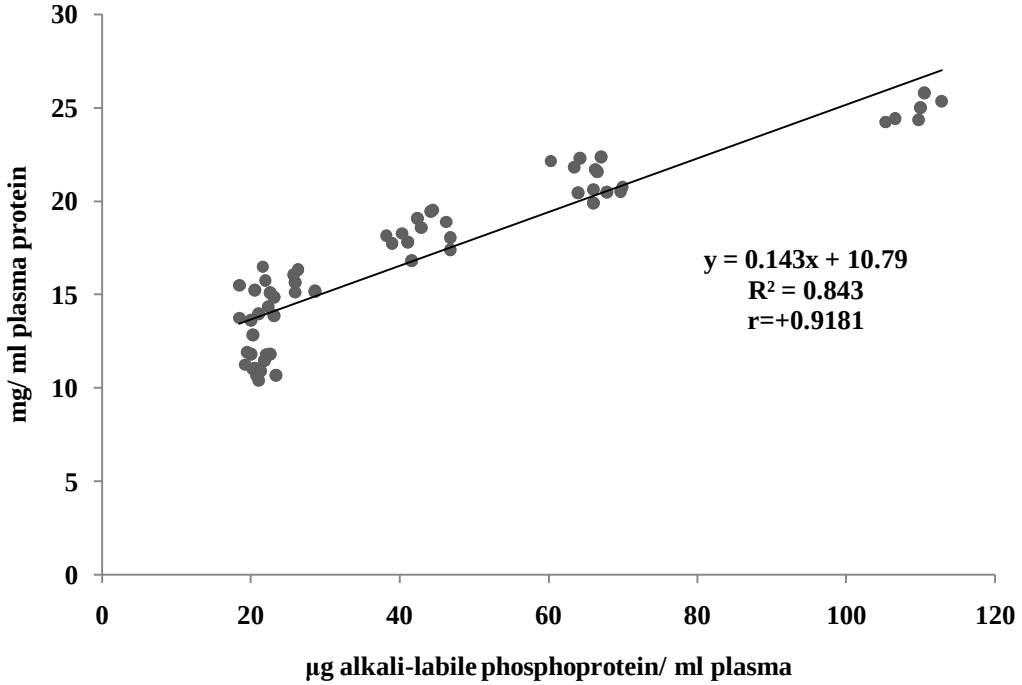


Figure 9: Correlation between plasma calcium and alkali-labile phosphoprotein exposed to chlordecone in female fish, *Pseudetroplus maculatus*

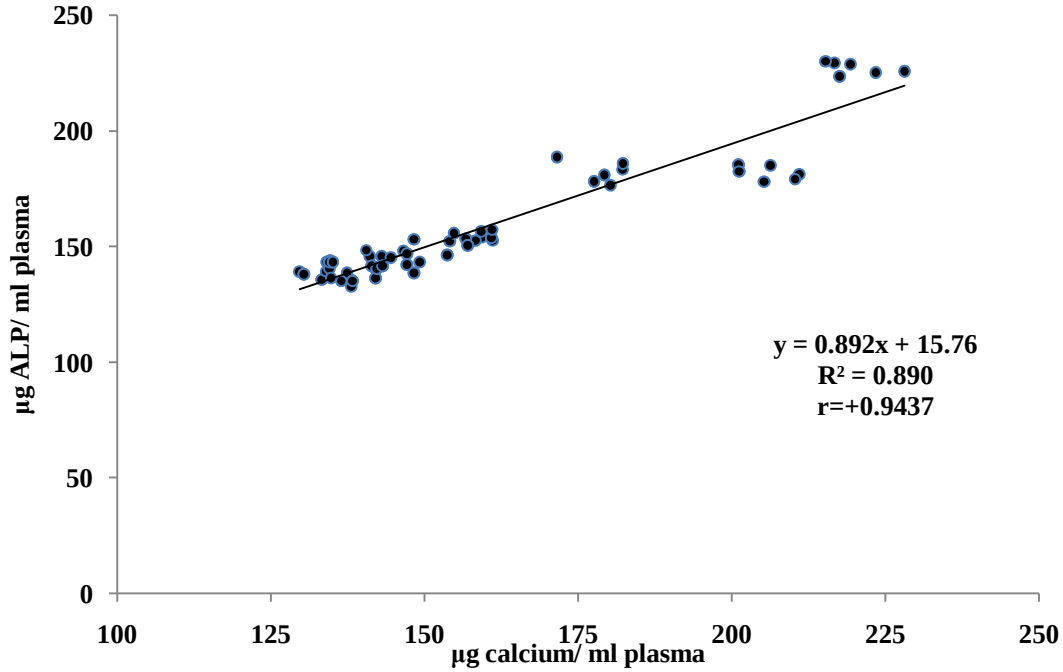


Figure 10: Correlation between calcium and plasma protein exposed to chlordecone in the female fish, *Pseudetroplus maculatus*

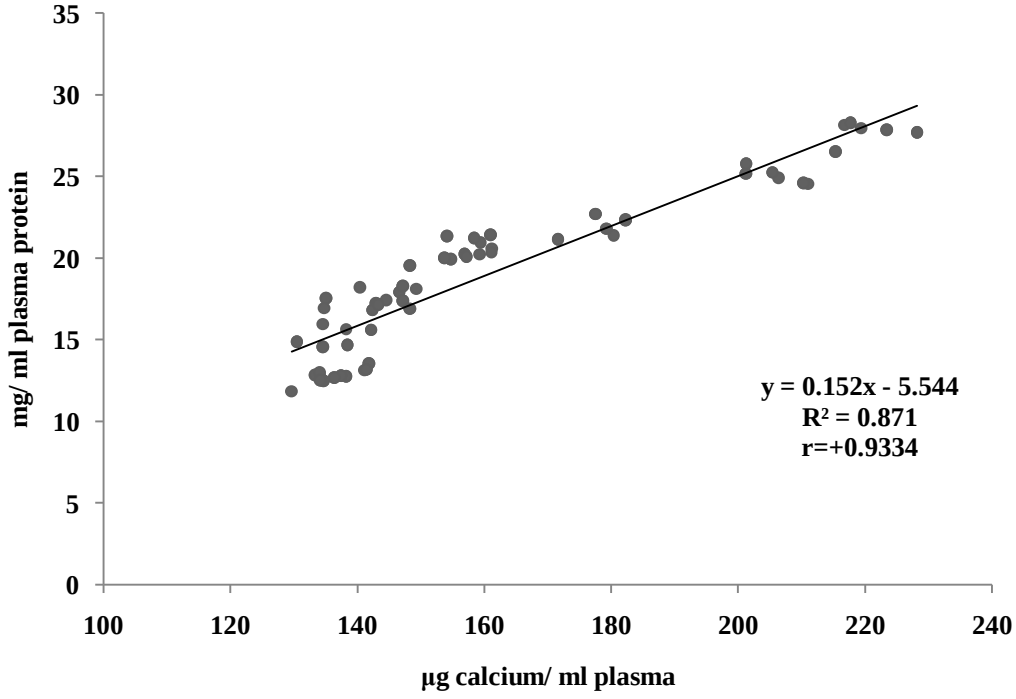


Figure 11: Correlation between alkali-labile phosphoprotein and plasma protein exposed to chlordecone in the female fish, *Pseudetroplus maculatus*

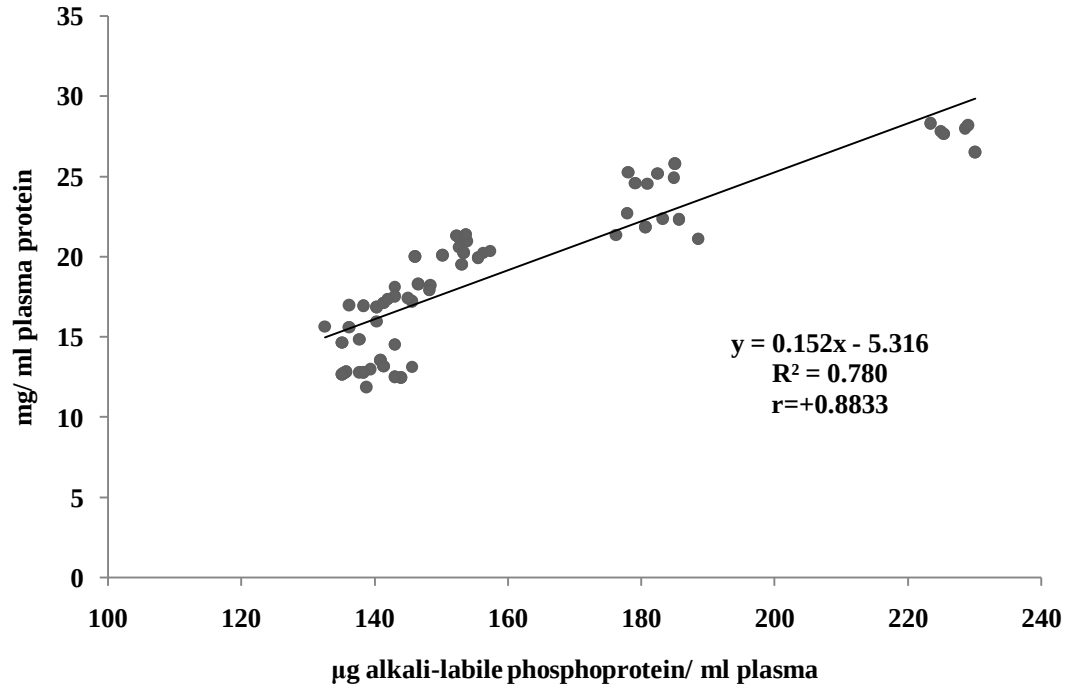


Figure 12: Short-term effect of chlordecone exposure on gonadosomatic index of the fish, *Pseudetroplus maculatus*

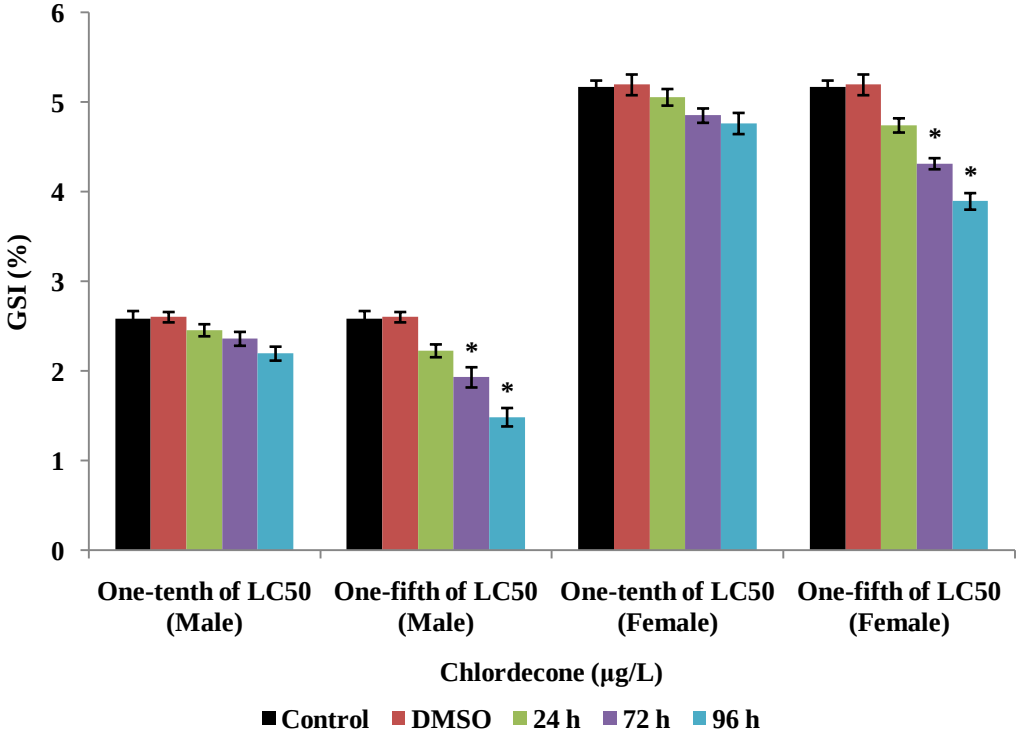


Figure 13: Long-term effect of chlordecone exposure on the gonadosomatic index of the fish, *Pseudetroplus maculatus*

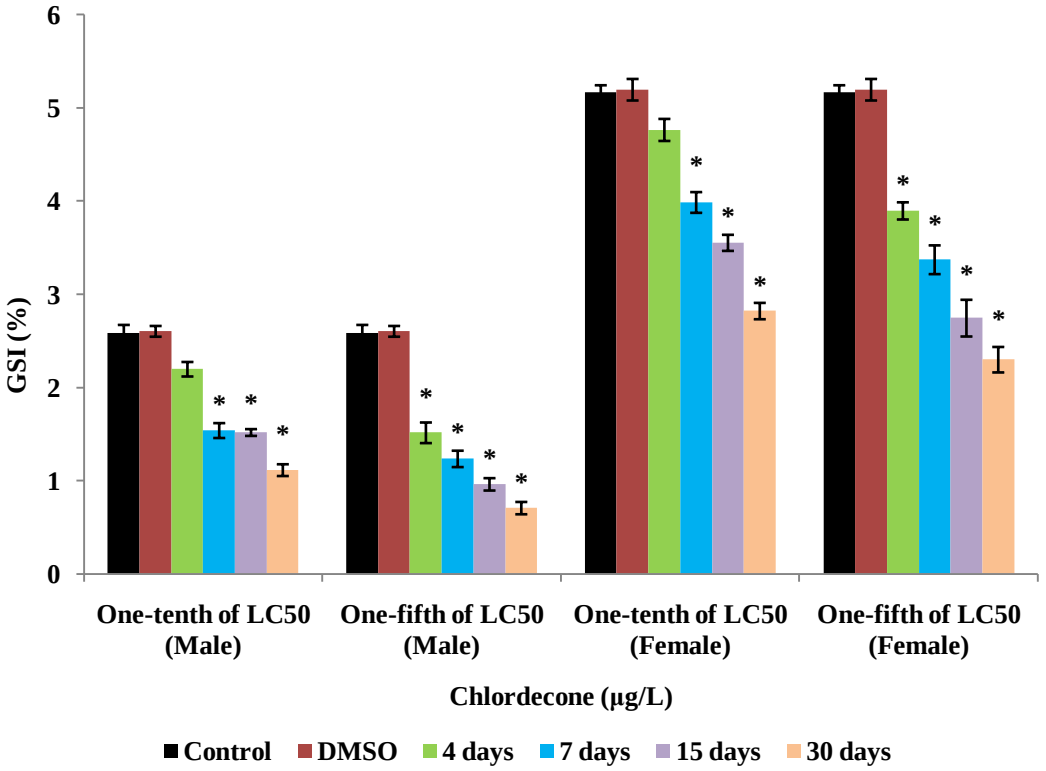


Figure 14: Effect of chlordecone on the activity of 3β-hydroxysteroid dehydrogenase in the testis of the fish, *Pseudetroplus maculatus*

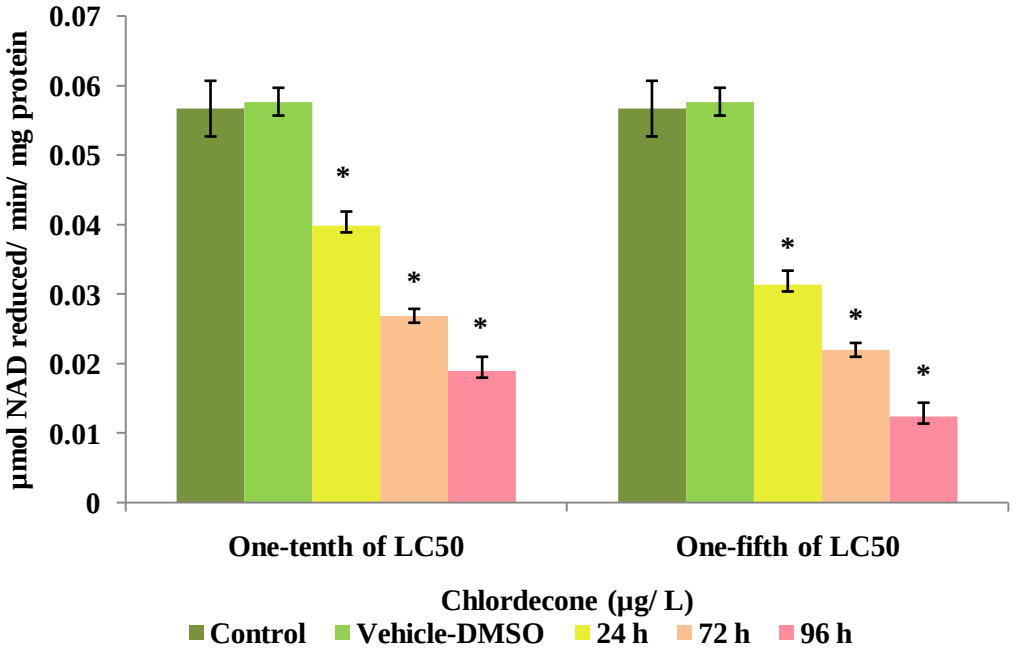


Figure 15: Effect of chlordecone on the activity of 17β-hydroxysteroid dehydrogenase in the testis of the fish, *Pseudetroplus maculatus*

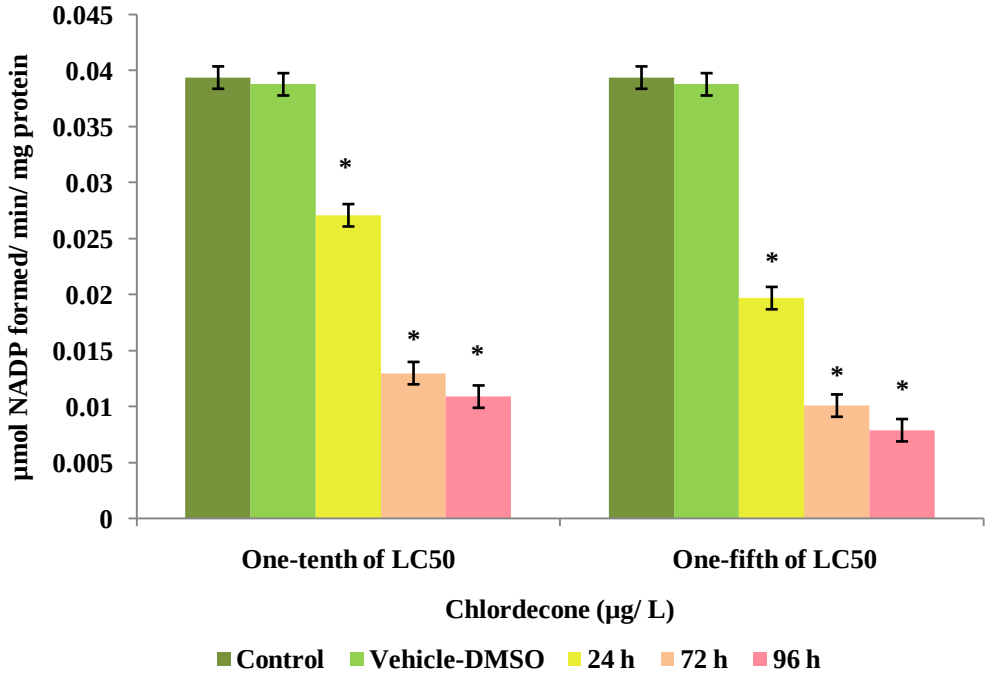


Figure 16: Effect of chlordecone on the activity of 3β-hydroxysteroid dehydrogenase in ovary of the fish, *Pseudetroplus maculatus*

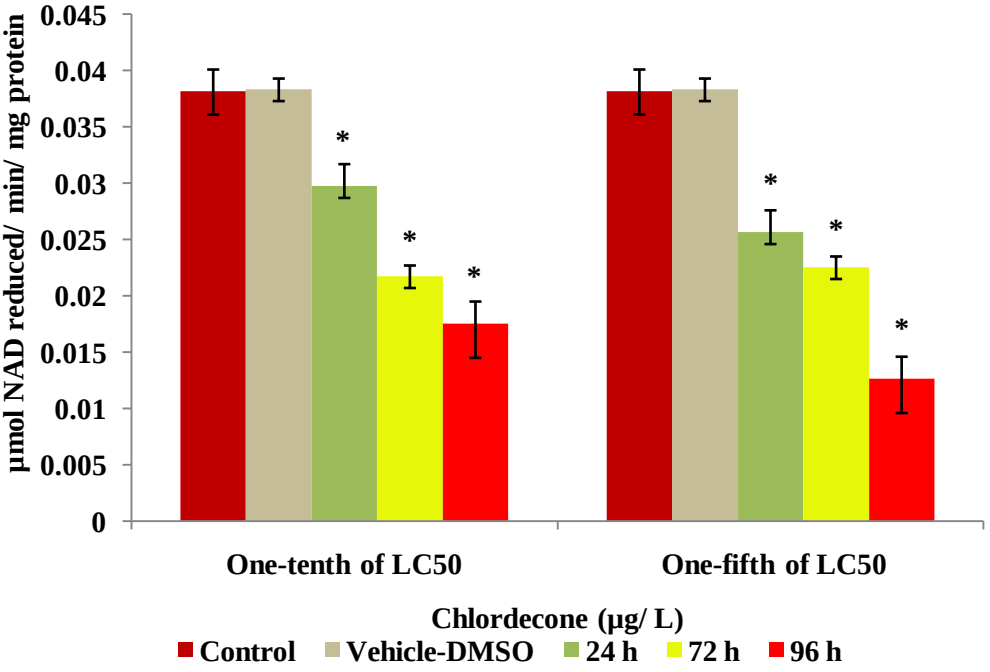


Figure 17: Effect of chlordecone on the activity of 17β-hydroxysteroid dehydrogenase in ovary of the fish, *Pseudetroplus maculatus*

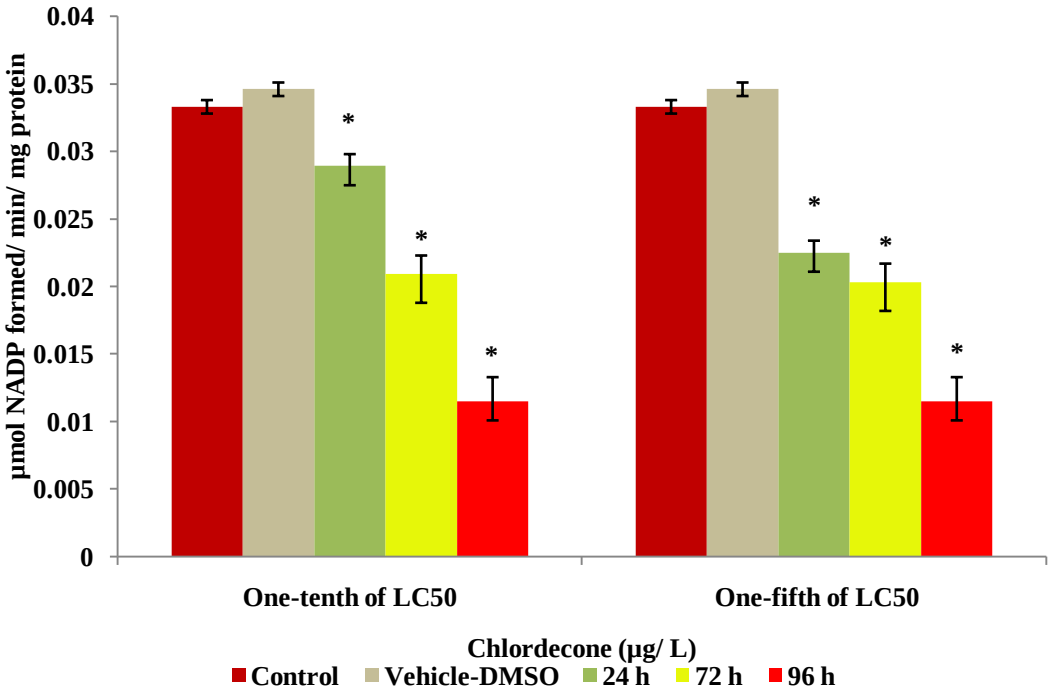


Figure 18: Effect of chlordecone on the sperm concentration in the fish, *Pseudetroplus maculatus*

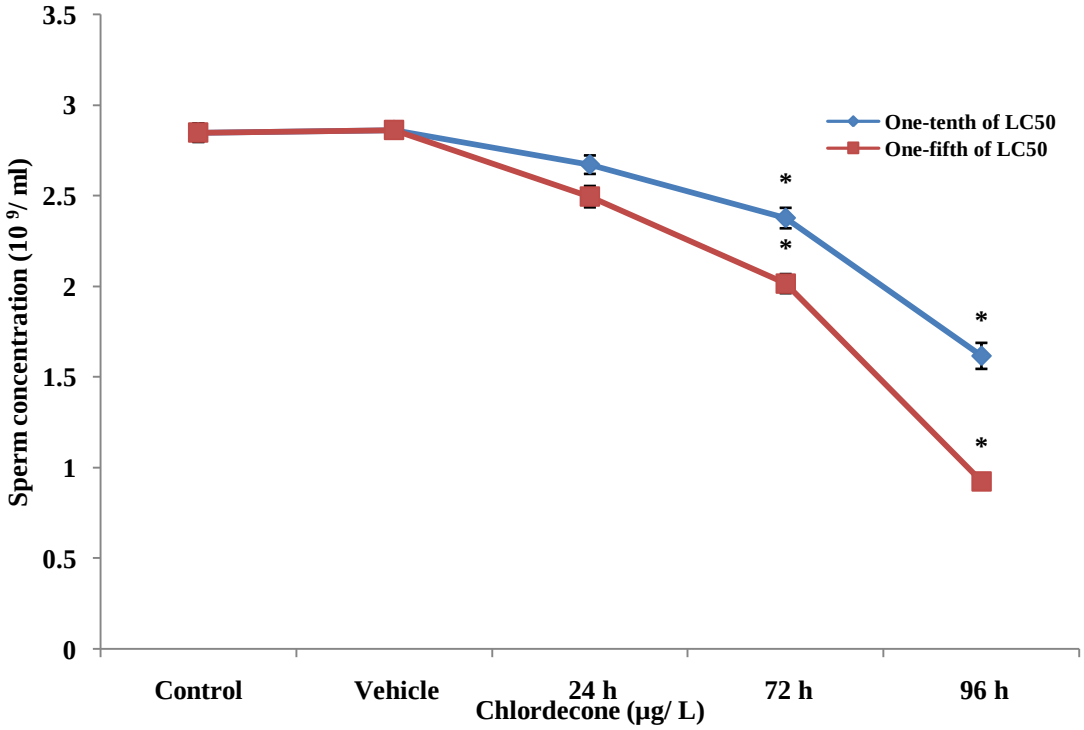


Figure 19: Effect of chlordecone on sperm viability in the fish, *Pseudetroplus maculatus*

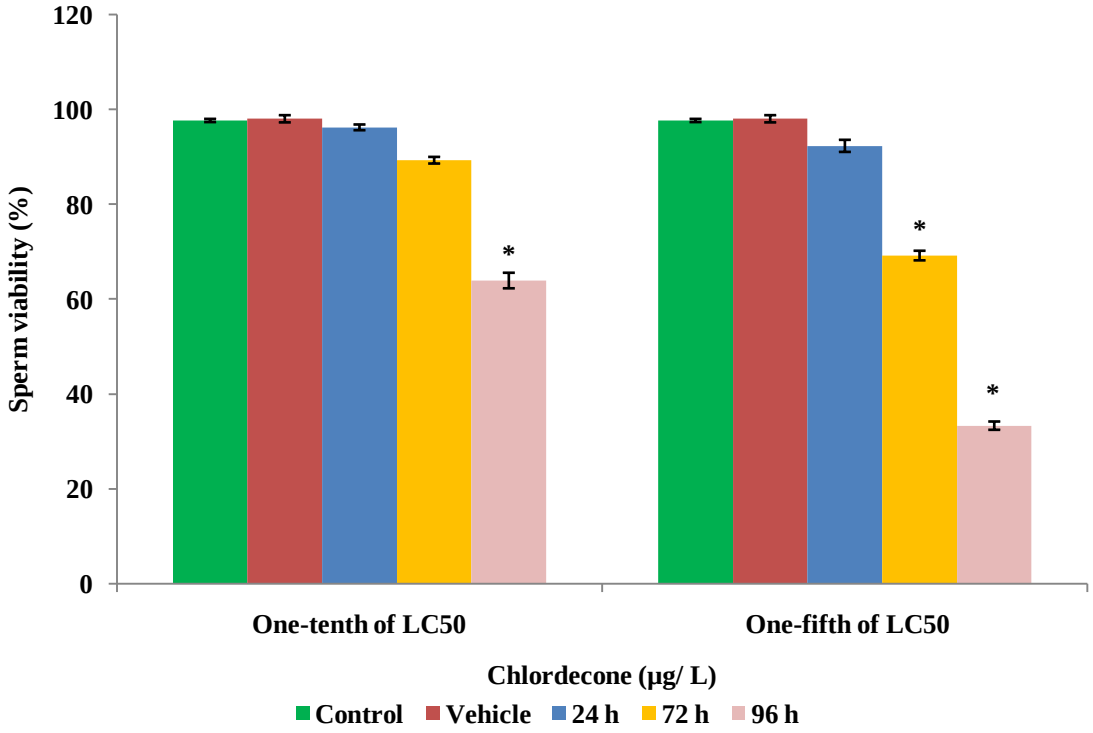


Figure 20: Effect of chlordecone on sperm motility in the fish, *Pseudetroplus maculatus*

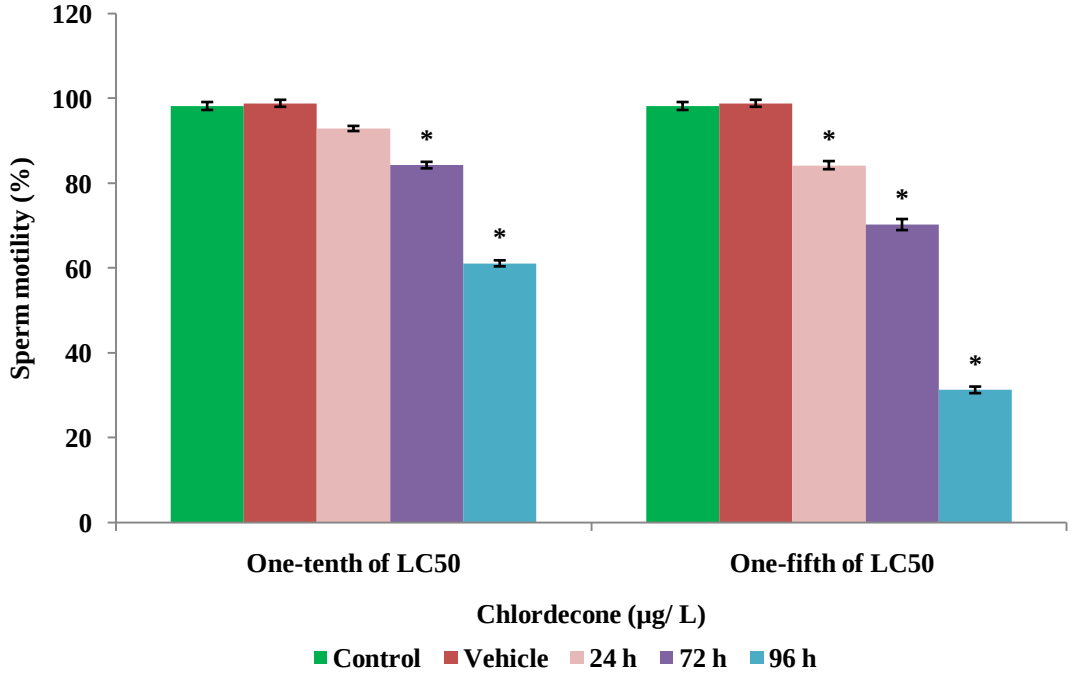


Figure 21: **A-Control and B-Vehicle (DMSO) showing viable sperm stained with Eosin; C-Chlordecone (3.5 µg/L) treated for 24 h showing few non-viable sperms(→); D and E-Chlordecone (3.5 µg/L) exposed for 72 and 96 h showing non-viable/dead sperm with enlarged head and absence/ reduced sperm tails (→).**

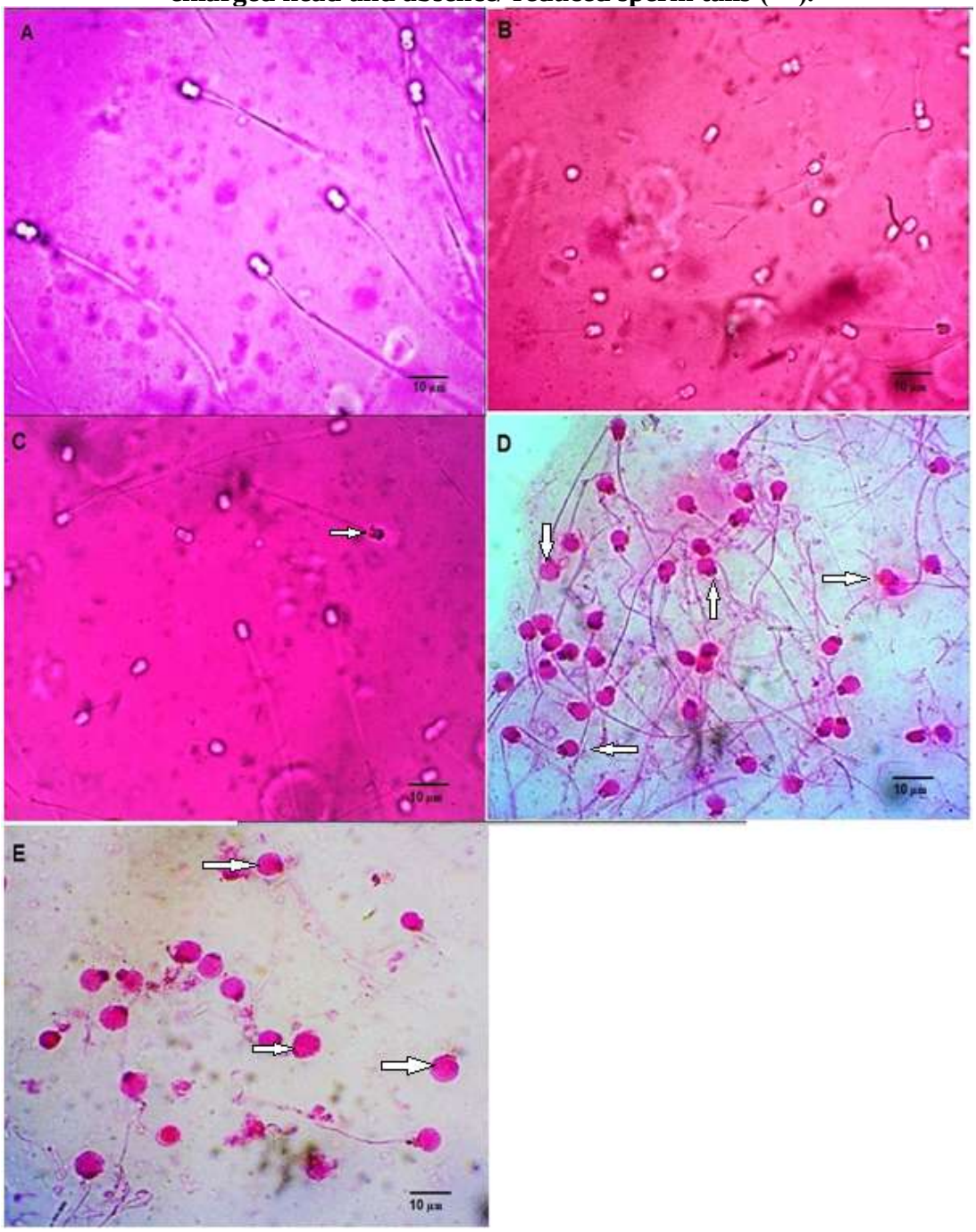
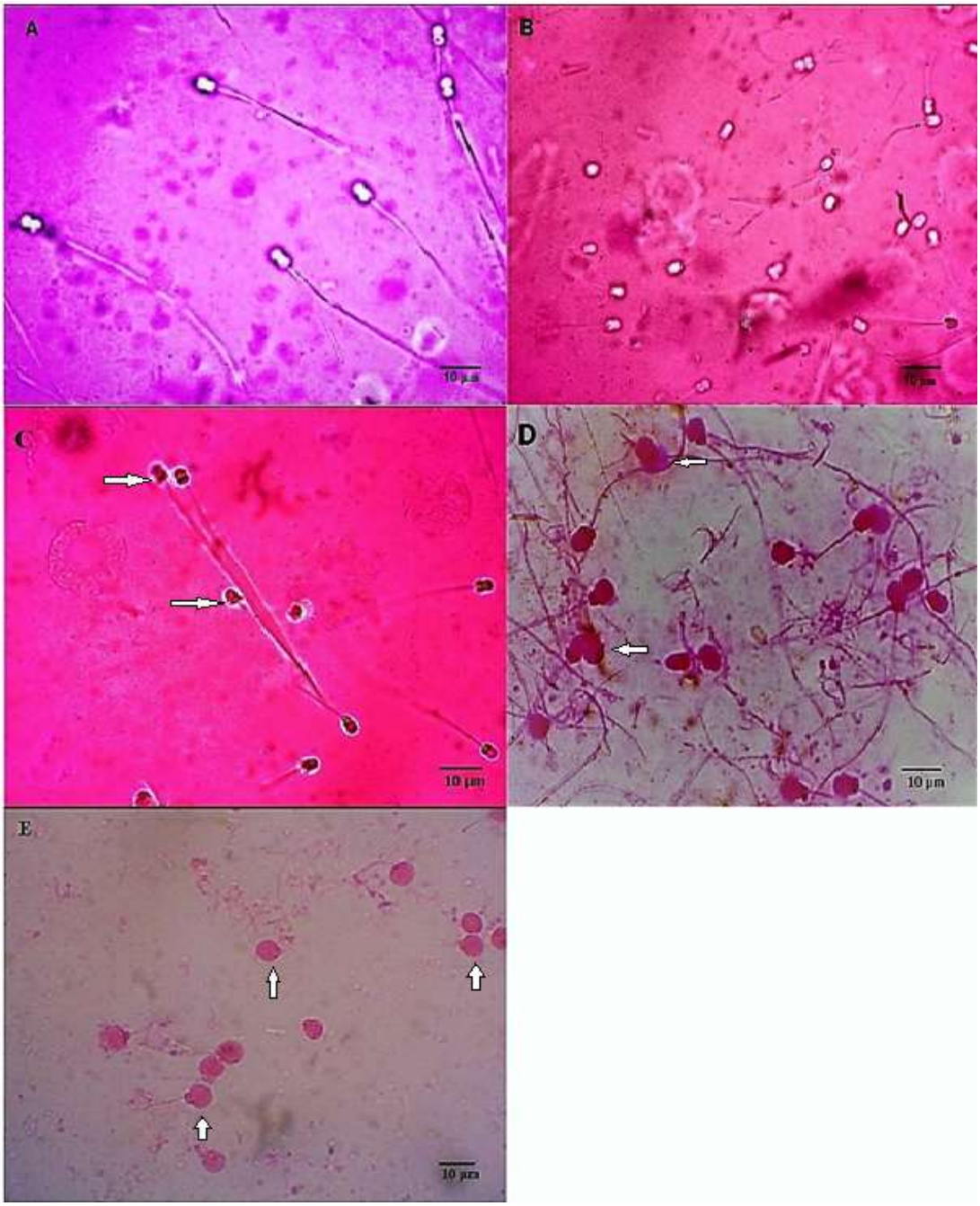


Figure 22: **A-Control and B-Vehicle (DMSO) showing viable sperm stained with Eosin; C, D and E-Chlordecone (7 µg/L) treated for 24, 72 and 96 h, respectively, showing non-viable sperm (→) having enlarged head (←) and absence/ reduced sperm tails (↑).**



Discussion

5.1 Effect of chlordecone on body weights and mucous deposition

The body weight of chlordecone-treated fish decreased in duration and concentration-dependent manner and this may be due to the necrosis or atrophy of tissues. Embryos of zebrafish when exposed to different concentrations of bisphenol-S showed decrease in body weight (Naderi *et al.,* 2014). Similarly, methoxychlor exposure caused a dose-dependent decrease in body weight in rainbow trouts (Krisfalusi *et al*., 1998). Hypersecretion of mucous observed after chlordecone exposure may be the primary defensive mechanism of the fish against the exposure to the environmental toxicant chlordecone. Mucous secretion is considered as efficient in seizing the toxic agents and thus helps to prevent the entry of these agents into the gills (Perry and Laurent, 1993). In fish, increased secretion of mucous all over the body surface prevents the entry of the toxicant into the body and thereby reduces the adverse effects of the toxicant (Mishra and Poddar, 2014). Mucous cells within the skin and gills were proliferated along with increased mucous secretions in *Clarias gariepinus* when exposed to ethanol extract of *Adenium obesum* stem bark (Abalaka *et al.*, 2015). This helps to clean the respiratory surfaces facilitating the removal of trapped toxicants from them (Chandra and Banerjee, 2004).

5.2 Effect of chlordecone on vitellogenin in male and female fish

Vitellogenin constitutes an important biomarker of endocrine disruption in fish (Hansen *et al*., 1998). The existence of vitellogenin in male fish is regarded as an indicator of exposure to estrogenic compounds in the environment and it is easily detected in the blood of exposed animal. Several studies have demonstrated the induction in the level of vitellogenin in males when exposed to xenoestrogens in the aquatic ecosystem. Male Japanese medaka, when exposed to octylphenol showed concentration-dependent induction of vitellogenin after 21 days (Gronen *et al*., 1999). Similarly, zebrafish exposed to ethinylestradiol induced the production of vitellogenin in dose-dependent manner (Orn *et al.,* 2003). Male fathead minnow, *Pimephales promelas,* inhabiting a lake in northwestern Ontario, Canada, which is contaminated with estradiol induce the production of vitellogenin along with

impairments in gonads, feminization of males and occurrence of intersex among males (Kidd *et al.,* 2007). Contamination of Mississippi River in United States by estrogenic effluents from sewage treatment plant is evidenced by the induction of vitellogenin, increased serum concentrations of estradiol and decreased levels of testosterone in both male and female walleye, *Stizostedion vitreum* (Folmar *et al.,* 2001). Thus, induction of vitellogenin is a useful tool to detect the presence of estrogenic chemicals in aquatic ecosystems.

The present study used indirect endpoints to measure the induction of vitellogenin in *Pseudetroplus maculatus* after chlordecone exposure, which involves assay of plasma calcium concentration, alkali-labile phosphoprotein (ALP), plasma protein and gonadosomatic index. Exposure of the fish to chlordecone had a significant effect on all tested plasma parameters. Chlordecone exposure elevated the plasma protein concentrations in both male and female fish when compared with the control groups in concentration and time-dependent manner. Similar result has been observed in rainbow trout when exposed to 17α-ethinylestradiol (Verslycke *et al.,* 2002). Plasma ALP and calcium concentrations increased 2 to 3-fold levels after exposure to chlordecone in males when compared to the corresponding control groups and the increase was duration-dependent. In females, the plasma ALP and calcium levels increased only after 30 days of chlordecone (3.5µg/L) exposure and after 15 and 30 days of exposure at higher concentration (7µg/L) when compared with the controls. The significant increase in all measured plasma parameters indicates the estrogenic response of chlordecone in the exposed fish. Induction in plasma ALP concentration has been reported in chub, *Leuciscus cephalus*, inhabiting contaminated river sites across the Walloon hydrographical network of southern Belgium (Mayon *et al*., 2006). Moreover, plasma protein, ALP and calcium levels were significantly and positively correlated in both male and female fish after chlordecone exposure. Thus elevated level of plasma vitellogenin in male fish can be used as an indicator of reproductive dysfunction. This may lead to alterations in testicular structure, formation of intersex, biased sex ratio and reduced fertility in males (Flammarion *et al*., 2000; Yokota *et al.,* 2000; Jobling *et al*., 2006).

5.3 Effect of chlordecone on gonadosomatic index

Gonadosomatic index (GSI) is the simplest, easiest and sensitive measure to assess the gonadal dysfunction in fishes (Kime, 1995). It varies in both male and female fish during the reproductive cycle and is used as a relevant biological variable for assessing the reproductive health status of fish (Adams *et al.*, 1993). Studies have reported that several pollutants induced alterations in gonads involving reduction in gonadosomatic index along with morphological and histological variations in the gonads (Singh and Srivastava, 2015). Reduction in gonadosomatic index may be due to the suppression of normal seasonal growth of the testes, or atrophy and inhibition of testicular development at puberty in males (Milnes *et al.*, 2006). Chronic exposure to chlordecone decreased the relative weight of both testis and ovary of the fish in concentration and time-dependent manner. Similar results were reported in adult male guppies, *Poecilia reticulate*, exposed to estradiol and tert-octylphenol (Toft and Baatrup, 2001). Reductions in gonadosomatic index have also been observed in wild perch exposed to bleach kraft mill effluent in the Baltic Sea (Sandstrom, 1994). Thus, reduction in the relative gonadal weight after chlordecone exposure might be due to gonad-specific toxicity.

5.4 Effect of chlordecone on the activities of steroidogenic enzymes

Major steroidogenic enzymes involved in the biosynthesis of sex steroids are 3β and 17β-hydroxysteroid dehydrogenases which are expressed in the testis and ovary. Activities of these enzymes were decreased significantly in concentration and time-dependant manner in both testis and ovary of *P. maculatus* after chlordecone administration. 3β-hydroxysteroid dehydrogenase is mainly involved in the synthesis of progesterone and testosterone from pregnenolone and androstenediol whereas 17β-hydroxysteroide dehydrogenase is responsible for the synthesis of testosterone and estradiol from androstenedione and estrone, respectively. In gonads of fish, conversion of pregnenolone to testosterone is catalyzed by the enzyme, 3β HSD, cyp17 and 17β –HSD (Wang *et al*., 2016). Thus interference with sex steroid biosynthesis may result in impaired reproduction, alterations in sexual differentiation, growth, and also involved in the development of certain cancers in

reproductive tissues (Sanderson, 2006). One of the persistent organic pollutants, perfluorooctane acid is known to inhibit the activities of 3β and 17 β–HSDs in the rat Leydig cells (Zhao *et al*., 2010). Similar results were noticed in the testis of rats, when it is exposed to sodium arsenite (Jana *et al*., 2006). Another study has reported that when rat is treated with sodium arsenite showed a significant reduction in the activities of both steroidogenic enzymes in the ovaries and it was reversed with co-administration of L-ascorbate (Chattopadhyay *et al*., 2001). Hence, chlordecone induced alterations in the activities of the steroidogenic enzymes could have some profound effect on the steroid biosynthesis pathway in the testis and ovary of the fish. The effects studied represent one of the ecologically relevant indicators of endocrine disruption by chlordecone and the effects are found severe in males when compared to female fishes.

5.5 Effect of chlordecone on sperm parameters

Sperm function tests such as sperm concentration, sperm viability and sperm motility were evaluated in order to assess the effects of chlordecone at two sublethal concentrations (3.5 and 7µg/L) for 24, 72 and 96 h durations in the male reproductive system of *Pseudetroplus maculatus*. In normal condition, fish sperm restrict the movement within the body of animal and are motile only when released into the external environment. Therefore, it is highly susceptible to disturbances by environmental contaminants including endocrine disruptors. The adverse effect caused by the toxicants may be either direct or indirect, involving disruption of spermatozoa and testicular function, respectively. Direct exposure of sperm to contaminants induces impairments in the activation of sperm leading to decreased motility, velocity and viability, which finally affect the fertilizing potential (Hatef *et al.*, 2013). Whereas, indirect exposure lead to testicular damage by affecting the cellular architecture of testis associated with induction of oxidative stress (Dorval *et al.*, 2005; Perez-Cerezales *et al.*, 2009). In addition, it also affects the process of spermatogenesis and steroidogenesis, which ultimately reduce the quality of sperm (Kime and Nash, 1999).

The present study revealed that chlordecone exposure at two sublethal concentrations decreased the sperm concentration, viability and motility compared with the controls. Male fertility highly dependent on motility of sperm and thus the present results indicate that chlordecone reduced the fertility of male fishes. Similar results were reported in trout, when exposed to environmentally relevant concentrations of the endocrine disruptor, bisphenol A (Lahnsteiner *et al.,* 2005). Similarly *in vitro* exposure to mercury reduces the number of sperm and induced morphological abnormalities in sperm of *Gymnotus carapo* (Vergilio *et al.*, 2013). Chlordecone exposure also leads to infertility in males among the employees in the production factory, which is evidenced by significant reduction in sperm counts and formation of oligospermia (Marquardt *et al*., 1999). Administration of chlordecone in rats at 15-30ppm for 60 days decreased the sperm reserves in the cauda epididymis as well as motility and viability of epididymal spermatozoa without affecting sperm morphology and concentration in epididymal fluid (Linder *et al*., 1983). Decreased sperm functions may also be due to disruption of endocrine system, alterations in testicular steroidogenesis, spermatogenesis or degenerative changes in the gonad structure due to the toxicity of chlordecone.

The decrease in sperm motility and viability may be due to the induction of oxidative stress in gonads (Kao *et al.,* 2008). Similar to mammalian spermatozoa, fish spermatozoa also contain high level of polyunsaturated fatty acid, which is highly susceptible to oxygen induced damage mediated by lipid peroxidation (Vernet *et al.,* 2004; Zhou *et al*., 2006). An increase in reactive oxygen species caused induction of oxidative stress which has been linked to abnormal or damaged testicular function (Sayed and Ismail, 2017). Chlordecone exposure is known to generate reactive oxygen species and induction of oxidative stress in the testis and ovary of *Pseudetroplus maculatus* after short-term durations which is discussed in chapter 4.

Conclusions

1. Exposure to chlordecone induced vitellogenin production in male fish proving estrogenic effect of the compound.

2. Chlordecone treatment modified the process of steroidogenesis as indicated by the decrease in the activities of steroidogenic enzymes.

3. Chlordecone reduced the reproductive potential of male fish as evidenced by the alteration in sperm parameters as sperm concentration, viability and motility.

4. In brief, chlordecone is a potent estrogenic inhibitor of male and female reproduction in the fish, *Pseudetroplus maculatus*.

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CHAPTER 3

Hormonal changes induced by chlordecone in *Pseudetroplus maculatus*

Introduction

1.1 Endocrine regulation of reproduction in fish

Fishes use both environmental and endogenous signals for the regulation of successful reproduction. Environmental signals include temperature, salinity, pH, photoperiod, abundance of food, social interactions, climatic conditions as water current, tides and cycles of moon, weather cycles etc. (Stacey, 1984). Endogenous signals are the chemical messengers secreted from different endocrine glands that act through brain-hypothalamus-pituitary-gonad chain (Yaron and Levavi-Sivan, 2011). Both signals contribute equally for the appropriate growth of gonads, sexual maturation and reproduction in fish. As in other vertebrates, reproduction in fish is under the control of endocrine system based on hypothalamus-pituitary–gonadal (HPG) axis by the interaction of several hormones. Hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the endocrine cells of pituitary gland for the synthesis and secretion of gonadotropic hormones (GtHs), which in turn regulates the gonads for mediating reproductive processes (Arcand-Hoy and Benson, 1998). Pituitary of fish contains gonadotrophs (FSH/LH) cells, which release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones control testicular and ovarian functions such as spermatogenesis, spermiogenesis, oogenesis, vitellogenesis etc. for maintaining successful reproduction (Asimakopoulos, 2012).

1.2 Hypothalamic-pituitary-gonadal (HPG) axis

Hypothalamic-pituitary-gonadal axis includes hormones from hypothalamus, pituitary and gonads, where it functions together as a single system. The axis is known to control various processes such as development, reproduction and ageing in fish as in other animals. Imbalance in the secretion of hormones in the axis results in various local and systemic effects such as gonadal dysfunction, decrease in sperm count, motility, failure of egg production, infertility etc. Gonadotropin-releasing hormone, GnRH-expressing neurons found in the hypothalamus secretes a releasing factor called gonadotropin-releasing hormone (GnRH). It acts on the gonadotroph cells of adenohypophysis to release follicle-stimulating hormone and luteinizing hormone, which travel into the blood stream primarily to activate gonads. As in

other vertebrates, the functions of pituitary gland are directly under the control of hypothalamus by several neurological connections (Scott, 1987). In teleost fishes, adenohypophysis is directly innervated by neurosecretory fibres of hypothalamus (Peter *et al*., 1990). In females, FSH and LH primarily act on ovaries to produce estrogen and inhibin. In males, LH stimulates interstitial cells to produce testosterone, whereas FSH is involved in the regulation of spermatogenesis (Peter *et al*., 1990).

1.3 Gonadotropin releasing hormone (GnRH)

GnRH is a tropic decapeptide hormone secreted by the neurosecretory cells of hypothalamus to stimulate the production and secretion of gonadotropic hormones through GnRH receptor i.e., GnRH-R in the anterior pituitary gland (Goos, 1991). The mechanism of action is mediated through G-protein coupled receptor (GPCR) where several molecular variants of GnRH have been isolated and characterized from different fish species (Sherwood *et al.*, 1983; Somoza *et al.*, 2002). GnRH exists in three different classes as GnRH1, GnRH2 and GnRH3, which are encoded by different genes and distributed distinctly in the brain of fish (White and Fernald, 1998). GnRH is known to perform various functions in fish species, where GnRH1 has been associated with the control of growth hormone (Marchant *et al*., 1989) and prolactin secretion (Weber *et al*., 1997). GnRH2 play an important role in sexual behaviour and in controlling appetite, depending on the species (Volkoff and Peter, 1999; Kauffman and Rissman, 2004). GnRH3 is primarily found in teleost fish and are involved in nesting behavior (Yamamoto *et al*., 1997). Administration of GnRH either by injection or as pellet mixed with cholesterol has been shown to induce oocyte maturation, ovulation, and spawning in Atlantic salmon (Crim and Glebe, 1984). The receptors of GnRH were distributed throughout the brain right from olfactory bulb to the medulla, as well as in the pituitary, which stimulate the secretion of gonadotropic hormones. GnRH receptors have evolved in variety of forms and therefore, the physiological role is promiscuous. However, growing evidence suggest the role of GnRH in the control

of pituitary gland for the secretion of follicle stimulating hormone (FSH) and leutinizing hormone (LH) (Gothilf *et al*., 1996).

1.4 Gonadotropic hormones (GTHs)

In response to GnRH, anterior pituitary secretes gonadotropins to stimulate the gonads for the production and release of sex steroid hormones. Gonadotropins are heterodimeric glycoprotein hormones consisting of two subunits namely GTH-α and GTH-β subunits (Kawauchi *et al.,* 1989). Further studies revealed that alpha-subunit release thyroid-stimulating hormone and beta-subunit secretes follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Chyb and Breton, 2000). Gonadotropins exert their activities through G-protein coupled receptor pathway. The role of these hormones mediating the reproductive cycle of fish has been studied in many species (Gen *et al.,* 2003; Shi *et al.,* 2015). In fish, FSH is the predominant hormone involved in the stimulation of development of ovary, vitellogenesis and in testicular spermatogenesis during early gamete formation. LH is widely involved in the late gamete maturation leading to ovulation or spermiation. Therefore, FSH producing cells are more prominent in the pituitary gland of immature fish and also in fish undergoing vitellogenesis, while LH-producing cells are found numerous only at the end of oocyte maturation (Naito *et al*., 1991).

Gonadotropins are thus an important key element for regulating the maturation of gonads, thus the determination of the level of gonadotropins in serum is an important measure in assessing the reproductive physiology of fish. Also the pattern of secretion of gonadotropins varies during the reproductive cycle (Nagahama *et al*., 1994). The primary targets of gonadotropins are gonads where it controls two main processes namely gametogenesis and steroidogenesis. In testis, LH stimulates Leydig cells for the production of sex steroids especially, testosterone by activating the LH receptor (LH-R) whereas FSH is expressed in Sertoli cells to promote spermatogenesis along with growth and development of germ cells by stimulating the FSH receptor (FSH-R) (Schulz and Miura, 2002). FSH and LH also functions together for the control of the process of steroidogenesis in the follicles by

regulating the expression of steroidogenic enzyme and steroidogenic acute regulatory protein (StAR) genes (Nakamura *et al.*, 2005).

In ovary, FSH stimulates the theca cells for the production of steroid hormone, testosterone, which in turn undergoes aromatization and converted to female specific sex hormone, 17β-estradiol in the granulosa cells. FSH also controls the uptake of yolk protein vitellogenin in vitellogenic follicles of the ovary (Fard *et al.*, 2013). Meanwhile, LH is also involved in the regulation of early and late oogenesis in females (Gen *et al.*, 2003). In addition, LH also mediates the production of progestins, which are maturation inducing hormone, maintaining the composition of the seminal plasma (Schulz and Miura, 2002). The interplay of gonads, pituitary and hypothalamus are important to regulate the biosynthesis and secretion of pituitary gonadotropins. FSH and LH act on gonads for the secretion of male and female specific sex steroid hormones, which in turn act on hypothalamus and pituitary either positively or negatively for the synthesis and secretion of FSH and LH.

1.5 Sex steroid hormones

In vertebrates, sex steroid hormones play important roles at all stages of the reproductive cycle. Three major classes of sex steroid hormones namely estrogen, androgen and progestins are chiefly produced in gonads. Major gonadal steroids in fish are 17β-estradiol (E2), 11-ketotestosterone (11-KT) and 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) or progestin, which are critical for the development and differentiation of gonads, regulation of gametogenesis, vitellogenesis, modification of reproductive behaviour and fertility in order to ensure successful reproduction in fishes (Nagahama, 2000). Estradiol is the major sex steroid mediating the female reproductive events, which is secreted by both the female gonads and inter-renal tissues. Under the influence of gonadotropins, the thecal cells of ovary produce testosterone, which traverse the basal lamina and enter into the granulosa cell layer where aromatase enzyme converts testosterone to estradiol (Kagawa *et al.*, 1985). The ability of the thecal cells to produce testosterone increase gradually during the growth of oocyte and its production reach maximum during post vitellogenic period.

Activity of aromatase enzyme in the granulosa cells also increase during vitellogenesis and gradually decrease when the oocyte undergoes maturation in response to gonadotropins (Kanamori *et al.,* 1988). Similarly, production of estradiol increases during oocyte growth and rapidly decreases in connection with oocyte maturation (Kagawa *et al.,* 1983). In addition, estradiol also stimulates the liver cells to produce vitellogenin, the precursor of yolk protein and is secreted into the blood. Within the blood it forms a complex with Ca^{2+}, sequestered by the ovarian follicles and acts as the source of nutrients required for development of the embryo (Nagahama *et al.,* 1994). Thus at the time of vitellogenesis, the concentration of estradiol is very high in the blood stream. There is evidence that a minimum level of estradiol is produced in the testis and the vitellogenin genes is also present in males, but in an inactive form (Canapa *et al*., 2006).

In teleost, the most potent androgen is 11-ketotestosterone, which is produced by hydroxylation of testosterone mediated by cytochrome P450 11β-hydroxylase ($P450_{11\beta}$) and its subsequent oxidation with the help of 11β-hydroxysteroid dehydrogenases (11β-HSD) (Melamed and Sherwood, 2005). Within the seminiferous tubules, it is essential to drive the process of spermatogenesis and also participate in the feedback regulation of brain and pituitary (Okuzawa, 2003).

Progestins are another important sex steroid hormones involved in reproduction. It is produced by the hydroxylation of testosterone within the liver, mediated by 20β–HSD. The major progestin in teleosts includes 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), which is also called maturation inducing steroid (MIS) hormone. It stimulates initiation of follicular maturation, final oocyte maturation, maturation of sperm, spermiation and ovulation in fishes (Miura *et al.,* 1992; Kazeto *et al.* 2011; Yaron and Levavi-Sivan, 2011). It is essential for initiating the process of meiosis in both spermatogenesis and oogenesis (Miura and Miura, 2008). Additionally, the DHP increase the pH of seminal plasma for facilitating the motility of sperm by activating the enzyme carbonic anhydrase in the seminal plasma (Miura *et al.,* 1992) and also induces sperm hydration (Ueda *et al.,* 1985). The action of sex steroids is also mediated by nuclear receptors, which act as ligand-dependent transcription factors within the nucleus (Aranda and Pascual,

2001). In most of the fishes, two nuclear estrogen receptors namely ER-α and ER-β, one androgen receptor (AR) and two progesterone receptors are found showing differential tissue-specific expression patterns (Thomas *et al*., 2006).

Similar to sex steroids, thyroid hormone also plays a significant role in reproduction and functions under the control of hypothalamus and pituitary. It is essential for development of gonads, gametogenesis, spermiation and ovulation and thus thyroid disruption usually affects reproductive processes or its outcome (Cyr and Eales, 1996; Arcand-Hoy and Benson, 1998). Thyroid stimulating hormone (TSH) is a glycoprotein hormone synthesized and secreted from adenohypophysis named as thyrotropin. Production and release of thyrotropin is under the control of thyroid releasing hormone, secreted by hypothalamus (Jennings *et al.*, 1990). TSH consists of α and β subunits and it stimulates the thyroid gland to secrete thyroid hormones (Pelkkikangas *et al*., 2004). Teleost fishes do not have an organized pair of thyroid glands as in other vertebrates. Thyroid follicles are found dispersed individually or in clusters, among afferent branchial arterioles of the ventral region of the pharynx. But, among vertebrates, including fish, the basic structure and function of the thyroid follicle is conserved (Paris and Laudet, 2008).

From the light of the above view points, it is clear that sex steroid hormones are important regulatory hormones, especially with regard to reproduction. Possibly the exposure to environmental contaminants having estrogenic properties can be expected to have significant consequences in the synthesis, secretion and regulation of sex hormones. Disruption of sex steroid hormones induced by chlordecone exposure and its consequences on male and female reproduction of the fish, *Pseudetroplus maculatus* is discussed in this chapter.

Review of Literature

Over recent years a wide range of endocrine disrupting chemicals are known to mimic the natural estrogenic hormones and exert the adverse effects on endocrine system. These chemicals are present in the water, sediments as well as in air, which can enter through food chain to aquatic animals and potentially cause serious effects on various physiological activities, especially on reproduction. Reproduction represents an ecologically relevant indictor of endocrine disruption in fishes (Arcand-Hoy and Benson, 1998). Most of the pollutants disrupt the endocrine function of reproduction by acting on hypothalamus, pituitary or gonad and bind directly to the hormone receptor (Mattison and Thomford, 1989). A wide range of pollutants are known to affect development, growth, differentiation and functioning of gonads, quality of gametes, rate of fertilization, induction of vitellogenesis in males, occurrence of intersex and sex reversal which ultimately results in impaired development, infertility and decreased population size of the exposed species (Pait and Nelson, 2002).

Chlordecone, the environmental contaminant used in the present study is a suspected endocrine disruptor with estrogenic activity. It is a strong estrogenic contaminant when compared to other xenoestrogens and acts as competitive agonist for estrogen receptors, ER-α and β (Kuiper *et al.,* 1998). Chlordecone elicit strong estrogenic effects in the oviduct of immature Japanese quail along with alterations in hypothalamo-adenohypophyseal axis (McFarland and Lacy, 1969). Estrogenic effect of chlordecone is also reported in rat which is evidenced by earlier vaginal opening, persistent vaginal estrus, and anovulation during prenatal and postnatal periods (Gellert, 1978; Gellert and Wilson, 1979). Employees working in the chlordecone production company experienced reproductive dysfunction and neurotoxicity, which are also the outcomes of chlordecone intoxication (ATSDR, 1995). On contrary, some studies have reported that chlordecone has antagonizing effect of estrogen and affinity of chlordecone to the estrogenic receptors is approximately 5000-fold lower compared to estradiol (Eroschenko, 1981; Huang and Nelson, 1986). Thus the present study was designed to evaluate if chlordecone mimics estrogen or possess antagonistic effects in *Pseudetroplus maculatus* by the determination of several hormones involved in hypothalamo-pituitary-gonadal axis in the fish.

Endocrine disruptors can elicit their effects by different mechanisms that can affect hormone synthesis and transport, by binding to estrogenic receptors, interfering germinal cell maturation and functions. The estrogenic effects are either agonistic or antagonistic to natural estrogens thereby causing disruption in the production, secretion, transport and metabolism of natural hormones as well as alterations in production and functioning of hormone receptors (Rotchell and Ostrander, 2003). Thus endocrine disruptors can activate either estrogen receptor (ER) or androgen receptor (AR) leading to the binding of the chemicals to the receptors and enter the nucleus of the target cell to initiate transcription of target genes. The mechanism of action is facilitated by binding to estrogen response elements or androgen response elements in the upstream regulatory region of the estrogen/androgen receptor-regulated genes (Goksoyr, 2006). Cytochrome P450 aromatase (CYP19) is another important targets of endocrine disruptors, which is a crucial steroidogenic enzyme catalyzing the final, rate-limiting step in the conversion of androgens to estrogens (Cheshenko *et al.*, 2008). Thus inhibition of aromatase leads to reduction of estradiol (E2), whereas increase of testosterone and induction of aromatase could result in elevated concentration of E2 and decreased concentration of testosterone in the blood plasma (Scholz and Mayer 2008). Usually, in males the level of estradiol is relatively low when compared with the female fish. However, endocrine disrupting chemicals released into the aquatic ecosystem has been shown to upregulate the activity of aromatase, which converts the male hormones to female hormones resulting in an increased level of estradiol and decreased level of testosterone (Lephart, 2015).

In fish, hypothalamo-pituitary-gonadal (HPG) axis plays an important role in maintaining successful reproduction and seasonal reproductive cycles in fish (Peter, 1991; Okuzawa *et al.*, 2003). Teleost HPG axis is considered as a novel graphical model in ecotoxicogenomics research on endocrine disrupting chemicals (Villeneuve *et al.*, 2007). The levels of gonadotropins and plasma sex steroids are considered as indicators of gonadal status and the concentrations may vary according to the reproductive cycle (Zohar and Mylonas, 2001). In male fishes, FSH predominates during spermatogenesis and LH predominates during spermiogenesis

and spermiation. Production of FSH is at peak during the early stages of gonadal development and vitellogenesis and LH predominates during the final oocyte maturation and ovulation in females (Yaron and Levavi-Sivan, 2011). Similarly, highest concentration of E2 was reported few weeks before spawning and it drops to basal level prior to ovulation in rainbow trout (Fostier *et al*., 1978). Prior to spawning period, a surge in testosterone and estradiol has been noticed in Eelpout, *Zoarces viviparus* (Larsson *et al*., 2002).

The HPG axis constitutes one of the major targets of endocrine disruptors and it is the most targeted pathways of the toxicants (Carr and Patino, 2011). It is a highly dynamic and active system extremely sensitive to environmental stressors, including pollutants. In recent years, several scientific studies have been conducted to study the effects of endocrine disruptors on HPG axis. Exposure to environmental contaminants can cause disruption of hormones in HPG axis, which in turn lead to reproductive abnormalities in fishes. Several studies have shown that various environmental contaminants have the potential role to alter the normal levels of different hormones in the blood stream either by mimicking, antagonizing or interfering with their normal hormonal activity (Pait and Nelson, 2002). Exposure to organochlorine pesticide hexachlorobenzene (HCB) and benzopyrene results in increased concentration of estradiol in Nile tilapia (Rodas-Ortiz *et al*., 2008). Whereas, low levels of gonadotropin, testosterone and estradiol has been observed in *Heteropneusts fossilis* when exposed to γ- hexachlorocyclohexane after 4 weeks (Singh and Canario, 2004). Bisphenol S has been shown to alter the balance of sex steroids in both male and female zebrafish, which is evidenced by the increased concentrations of E2 and decreased concentration of testosterone and greater E2/T ratio along with upregulation of aromatase gene as well as down-regulation of 17β-hydroxysteroid dehydrogenase genes (Ji *et al.,* 2013).

Studies have shown that 17α,20β-dihydroxy-4-pregnen-3-one (DHP) is known to alter the process of mitosis and meiosis, final maturation stage of spermatogenesis, regulation of spawning behaviours and also induced spermatogonial DNA synthesis in Japanese huchen (Amer *et al.,* 2001). High level of DHP has been reported in the blood of male rainbow trout throughout the period

of milt production (Baynes and Scott, 1985; Scott and Sumpter, 1989). Involvement of DHP during early stages of spermatogenesis, especially initiation of meiosis has been demonstrated in eel testis *in vitro* culture system (Miura *et al.*, 2006). Thus, changes in hormones involved in the initiation, processing and maturation of gonads particularly those regulating gametogenesis and vitellogenesis may have potential role as early warning indicators of toxic effect of pollutants in fish.

Sex steroids play an important role in gonadal development and sexual differentiation in fish (Scholz and Kluver, 2009). Hence, xenobiotics with steroid like activities are responsible for the incidence of gonadal intersex and other gonadal abnormalities in animals. Presence of intersexuality has been reported in a wild population of Mediterranean swordfish, *Xiphias gladius* L, in which the males possess vitellogenin in liver, previtellogenic oocytes and female germ cells within the testes, which confirms the prevalence of xenoestrogens in the aquatic ecosystem (De Metrio *et al.*, 2003). Both male and female fish exhibit gender-based sexual and reproductive behaviour during spawning, which is under the control of endocrine system especially androgen, prostaglandins from ovary and gonadotropin releasing hormone (Munakataand Kobayashi, 2010). Evidence reported that hormonal alterations could disrupt the reproductive behaviours leading to reduced fertilization rate in fishes (Soffker and Tyler, 2012).

Modifications in gonads and sexual behaviour are reported to severely affect the fecundity rate of fish by disturbing egg production, sperm count, sperm motility, fertility rate and hatching rate (Huang *et al.,* 2015). It has been reported that environmental contaminant such as barium chloride could disrupt production of steroid hormones, gene transcriptions in HPG axis, subsequently leading to decreased egg production and reduced hatching rates in zebrafish (Kwon *et al*., 2016). In another study, one of the endocrine disruptor bisphenol F is found to alter sex steroid hormones along with changes in egg production, hatching rate, and survival rate as well as histological impairments in gonads of zebrafish (Yang *et al.*, 2017). Thus, it was evidenced from the literatures that endocrine disruptors adversely affect the reproductive potential in fishes that ultimately lead to infertility and reduction in population size.

Materials and Methods

3.1 Chemicals

Quantification of hormones was performed by enzyme-linked immunosorbent assay (ELISA) using commercial kits obtained from Diagnostic System Laboratories, Inc. Webster, Texas, USA.

3.2 Sample collection

Based on the LC_{50}-96 h value of chlordecone, two sublethal concentrations - 3.5 μg/ L and 7 μg/ L (1/10th and 1/5th of LC_{50}) were selected and exposed to both male and female fishes for 4, 7, 15 and 30 days. Ten animals were maintained in both treatment concentrations, and also in positive and negative control groups.

At the end of every treatment period, by using small dip net, fish were caught gently one at a time without disturbances. Blood samples collected from control and treated groups at both sublethal concentrations were used for hormone assays. Blood was obtained from both male and female fishes by cardiac puncture method with the help of syringe and collected in separate clean microcentrifuge tubes. Blood was centrifuged at 1700*g* for 15 min at 4ºC to obtain serum and stored at -80ºC in microcentrifuge tubes for hormone analysis. Serum levels of TSH, FSH, LH, testosterone and estradiol were measured immediately using commercial ELISA kits. The assays were done strictly according to the procedure given along with the kits. For assaying serum hormones, all the samples and reagents were equilibrated with room temperature and were mixed thoroughly by gentle inversion prior to use. Standards, controls and unknowns were assayed in duplicate.

3.2.1 Quantitative determination of serum TSH

For assaying serum TSH, 100 μL of the standards, controls and unknowns were added to the appropriate wells. The wells were covered and incubated by shaking at a fast speed (500 - 700 rpm) on an orbital microplate shaker for 60 min at room temperature. The wells were aspirated and washed three times with wash solution using an automatic microplate washer and blotted dry. Then, 100 μL of antibody-enzyme conjugate solution was added to each well and were incubated by shaking at a fast speed on an orbital microplate shake for 30 min at room

temperature. The wash was repeated thrice and blotted dry, then 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) chromogen solution was added to each well and incubated, shaking at a fast speed for 10 min at room temperature. Stopping solution (100 μL; 0.2 M sulfuric acid) was added to each well and absorbance of the solution was read within 30 min using a microplate reader at 450 nm. A set of TSH standards was used to plot a standard curve from which the TSH concentrations in the unknowns were calculated and expressed as pg/mL.

3.2.2 Quantitative determination of serum FSH

For assaying serum FSH, 100 μL of the standards, controls and unknowns were added to the appropriate wells, incubated by shaking for 60 min at room temperature. Antibody-enzyme conjugate solution (100 μL) was added to each well, incubated with shaking speed of 500-700 rpm for 30 min at room temperature. The wells were aspirated and washed three times with wash solution, blotted dry and 100 μL of TMB chromogen solution was added to each well and incubated again for 10 min at room temperature. Stopping solution (100 μL; 0.2 M sulfuric acid) was added to each well and absorbance was read within 30 min at 450 nm. FSH concentrations in the unknowns were calculated from standard FSH values and expressed as pg/mL.

3.2.3 Quantitative determination of serum LH

For assaying LH in the serum, 50 μL of the standards, controls and unknowns were added to the appropriate wells. Antibody-enzyme conjugate solution (100 μL) was added to each well and incubated for 90 min at room temperature. The wells were aspirated and washed five times with the wash solution and blotted dry. Then, 100 μL of TMB chromogen solution was added to each well, incubated for 10 min at room temperature. Stopping solution (0.2 M sulfuric acid; 100 μL) was added to each well and absorbance was read at 450 nm. By using standard graph, the LH concentrations of the unknowns were calculated and expressed as pg/mL.

3.2.4 Quantitative determination of serum testosterone

For assaying serum testosterone, 50 µL of the standards, controls and unknowns were added to the appropriate wells and incubated with 100 µL of enzyme conjugate solution and testosterone-antiserum for 60 min at room temperature. Wells were aspirated and washed five times with wash solution using an automatic microplate washer and blotted dry. TMB chromogen solution (100 µL) was added to each well and incubated by shaking at a fast speed for 10 min at room temperature. Stopping solution (100 µL) was added to each well and absorbance was read at 450 nm. Testosterone standards were used to plot a standard curve, from which the testosterone concentrations of the unknowns were calculated and values are expressed as pg/mL.

3.2.5 Quantitative determination of serum estradiol

For assaying estradiol in the serum, 100 µL of standards, controls and unknowns were added to the appropriate wells of microplate. Estradiol-biotin conjugate solution (100 µL) was added to each well. The wells were incubated by shaking at a fast speed for 60 min at room temperature. Wells were aspirated and washed five times with the wash solution and blotted dry by inverting plate on absorbent material. Then, 200 µL of Streptavidin-enzyme conjugate solution was added to each well and incubated by shaking at a fast speed for 30 min at room temperature. The wells were aspirated and washed five times with the wash solution using an automatic microplate washer. TMB chromogen solution (100 µL) was added to each well and was incubated by shaking at a fast speed on an orbital microplate shaker, for 10 min at room temperature. Stopping solution (100 µL) was added to each well and absorbance of the solution in the wells was read within 30 min at 450 nm. From the estradiol standard graph plotted the concentrations of unknowns were calculated and expressed as pg/mL.

3.3 Statistical analysis

The results obtained in the experiment were analysed using statistical program SPSS 19.0 for Windows. The values of hormonal parameters were

expressed as Mean ± SD for n = 10 animals/ group. Analysis of variance (ANOVA) was performed to determine significant differences in sex hormones among different groups. Differences in mean values were analyzed by Duncan's Multiple Range test and the probability level for all statistical tests was set significant at $p<0.05$ against the control groups.

Results

4.1 Effects of chlordecone in serum hormone levels in male fish

In the present study, fishes exposed to DMSO (positive control) showed no significant changes in all serum hormone levels in male fish when compared with solvent-free control group (Figures 1-5). Male fish when exposed to chlordecone at 3.5µg/ L concentration showed significant ($P<0.05$) decrease in the level of TSH after 30 days of treatment (Figure 1). Chlordecone at one-fifth of LC_{50} concentration decreased the level of serum TSH significantly ($P<0.05$) after 15 and 30 days (Figure 1). Serum levels of FSH and LH showed no significant changes after 3.5µg/ L concentration of chlordecone in all treatment groups (Figures 2 and 3). However, at one-fifth of LC_{50} concentration, the level of FSH showed significant ($P<0.05$) decrease after 7, 15 and 30 days and the level of LH decreased significantly ($P<0.05$) only after 30 days (Figures 2 and 3). Serum testosterone level was found decreased significantly ($P<0.05$) after 15 and 30 days at 3.5µg/ L concentration of chlordecone (Figure 4). At higher sublethal concentration, the level of testosterone showed significant ($P<0.05$) reduction after 7 days onwards (Figure 4). The serum level of estradiol was found increased significantly ($P<0.05$) after 30 days at 3.5µg/ L concentration and after 15 and 30 days at 7µg/ L concentration when compared with the corresponding control groups (Figure 5).

4.2 Effects of chlordecone in serum hormone levels in female fish

In female fish, serum levels of TSH, FSH, LH, testosterone and estradiol remained unchanged in DMSO-treated group and found similar to the titre of negative control group (Figures 6-10). Serum TSH, FSH, LH and testosterone levels remained unchanged at one-tenth of sublethal concentration (Figures 6-9). However, when the concentration is increased to one-fifth the serum levels of TSH, LH and testosterone was found decreased significantly ($P<0.05$) after 30 days, however no significant changes were observed in the level of FSH (Figures 6-9). The level of estradiol was increased significantly at both concentrations after 30 days of exposure when compared with the control groups (Figure 10).

Figure 1: **Effect of chlordecone on the level of TSH in the male fish, *Pseudetroplus maculatus***

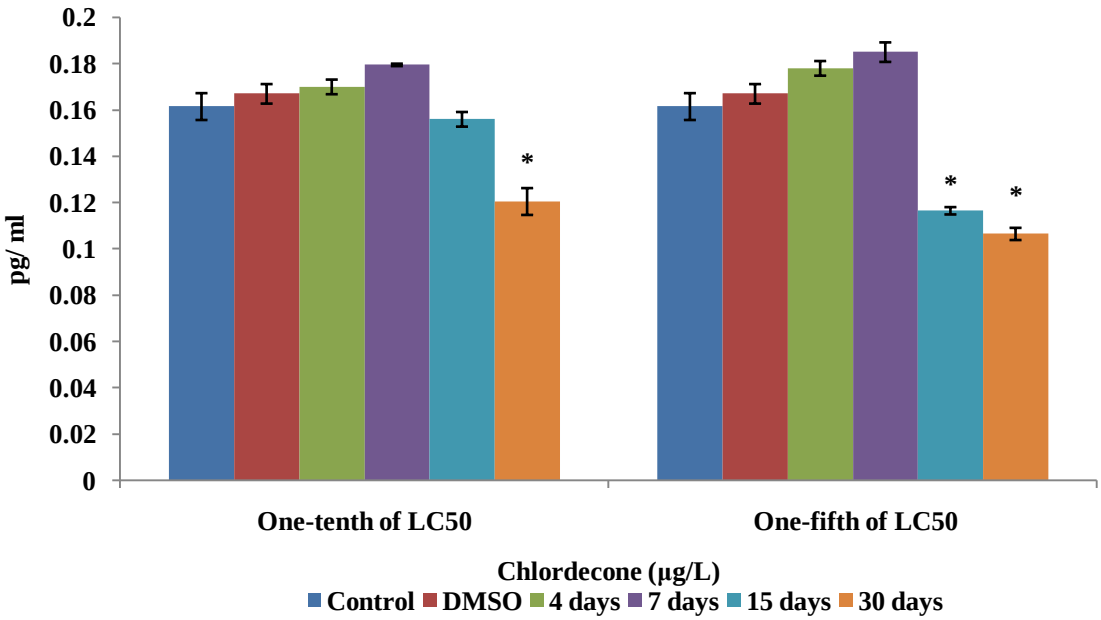


Figure 2: **Effect of chlordecone on the level of FSH in the male fish, *Pseudetroplus maculatus***

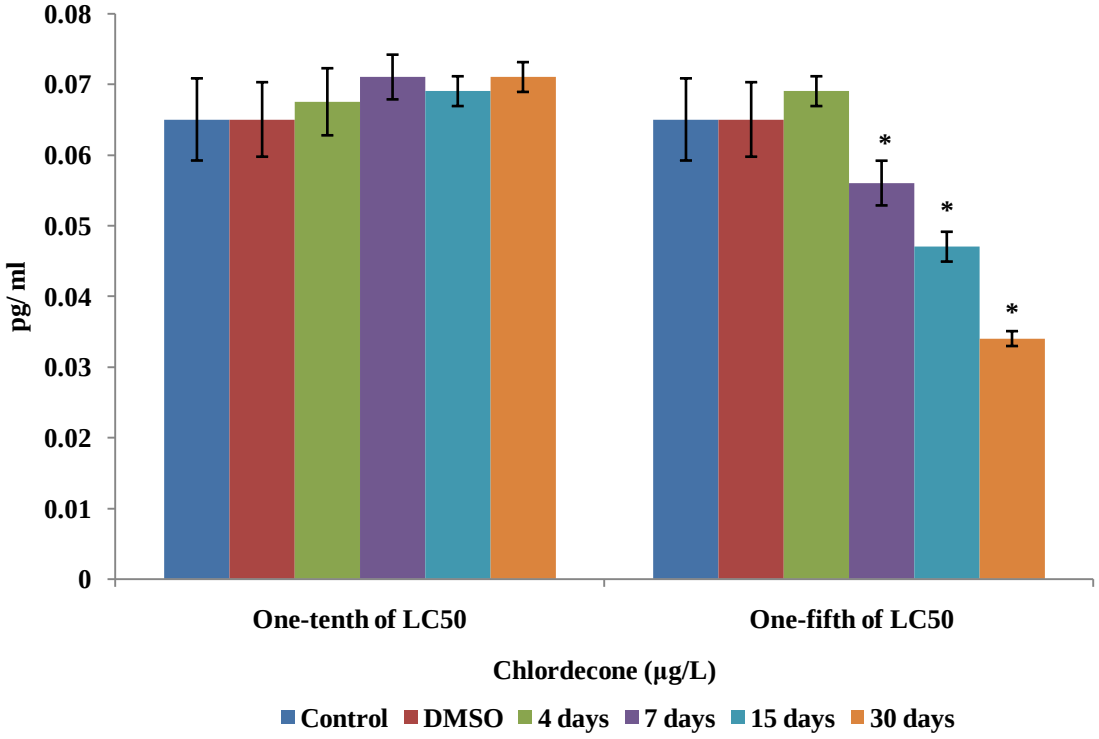


Figure 3: **Effect of chlordecone on the level of LH in the male fish, *Pseudetroplus maculatus***

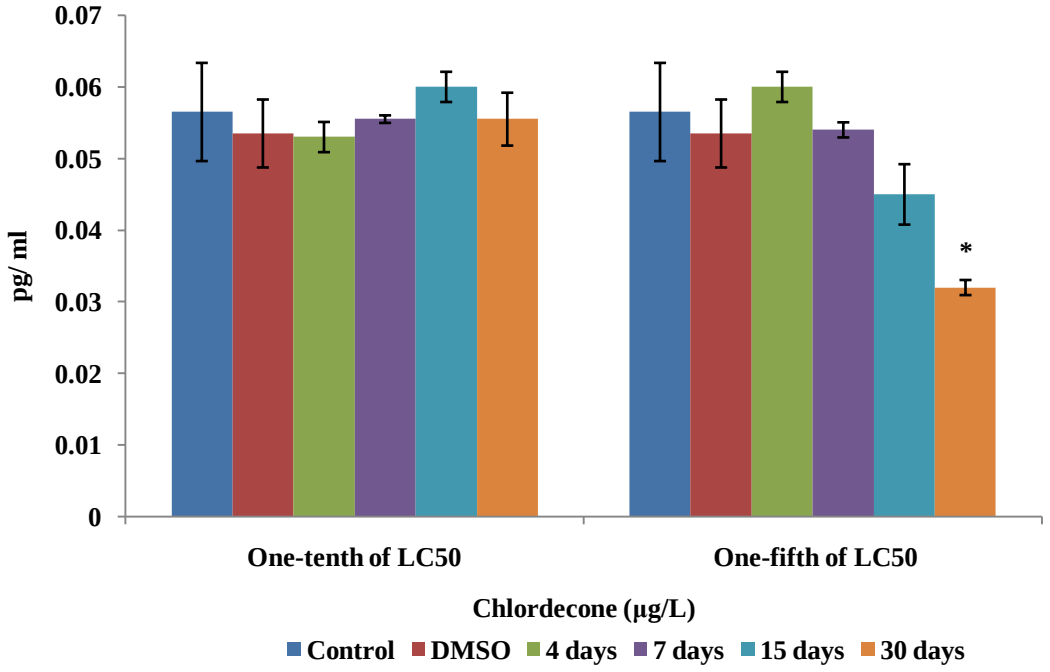


Figure 4: **Effect of chlordecone on the level of testosterone in the male fish, *Pseudetroplus maculatus***

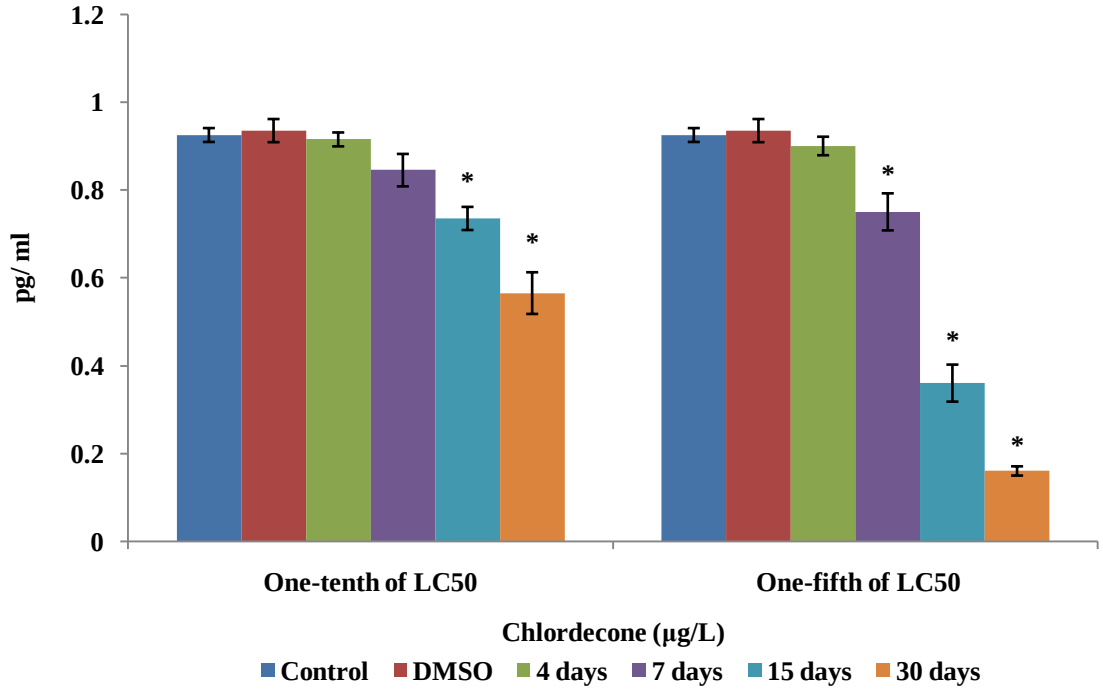


Figure 5: **Effect of chlordecone on the level of estradiol in the male fish, *Pseudetroplus maculatus***

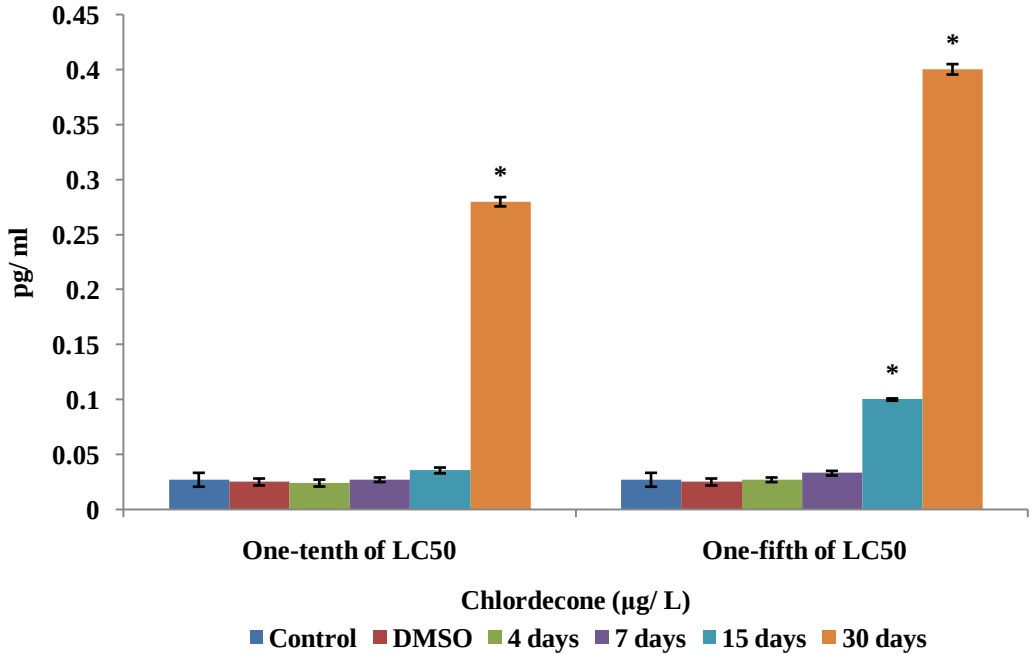


Figure 6: **Effect of chlordecone on the level of TSH in the female fish, *Pseudetroplus maculatus***

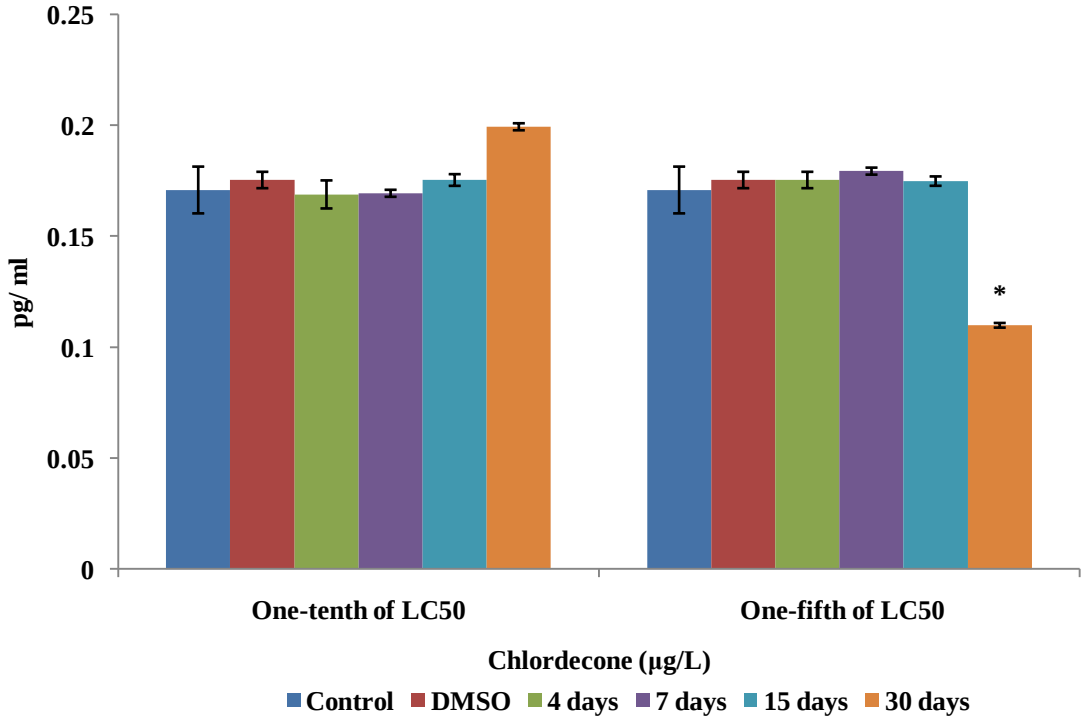


Figure 7: **Effect of chlordecone on the level of FSH in the female fish, *Pseudetroplus maculatus***

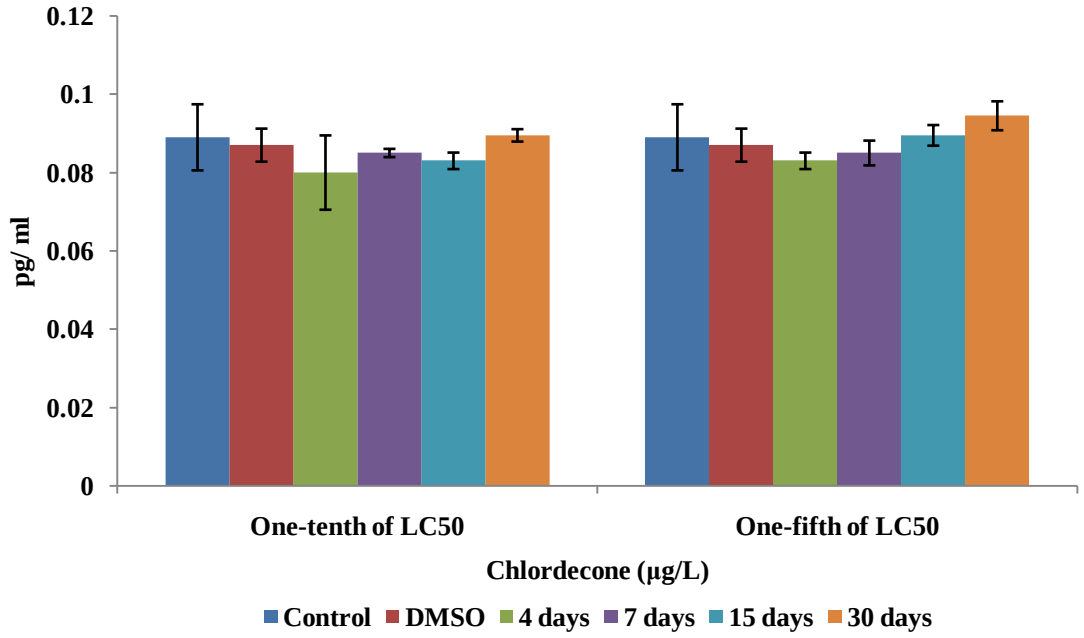


Figure 8: **Effect of chlordecone on the level of LH in the female fish, *Pseudetroplus maculatus***

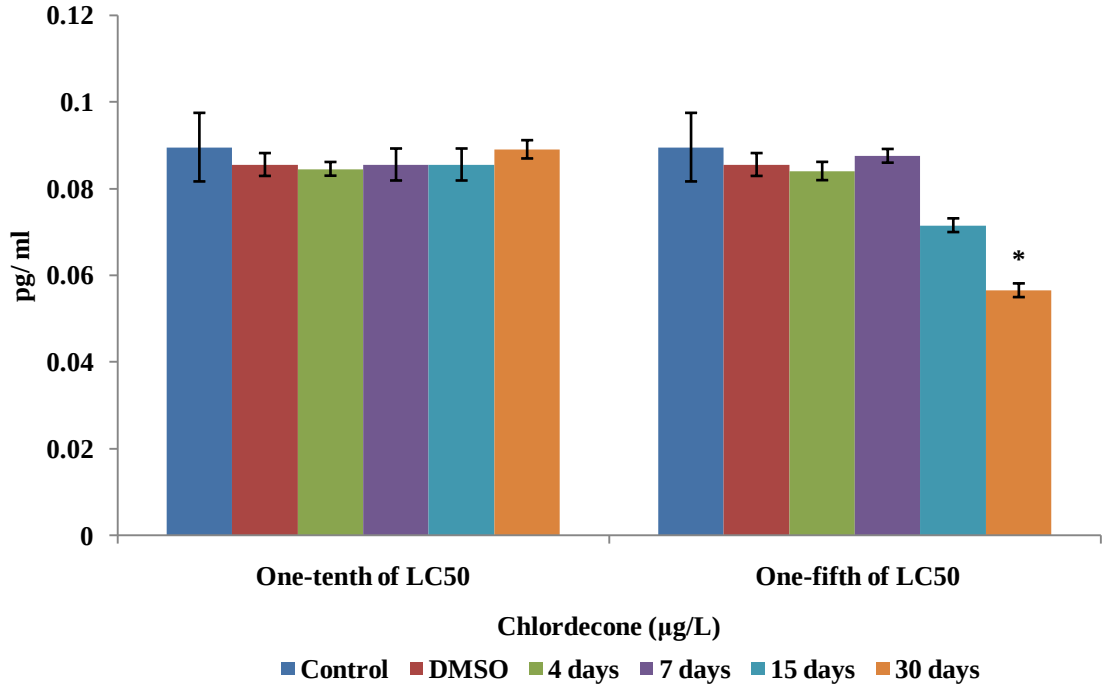


Figure 9: **Effect of chlordecone on the level of testosterone in the female fish, *Pseudetroplus maculatus***

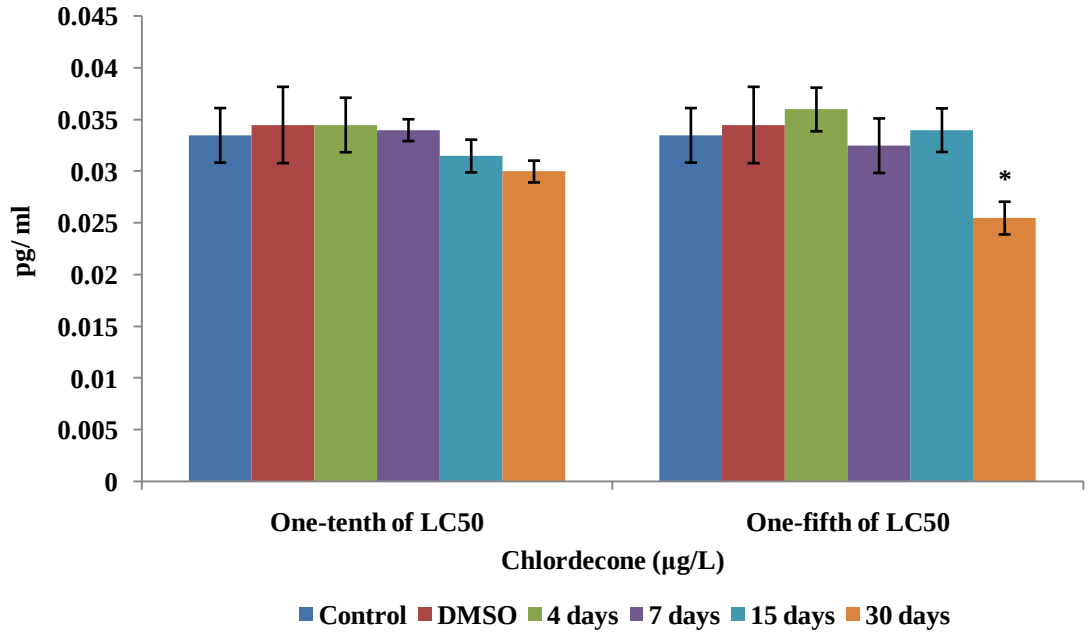
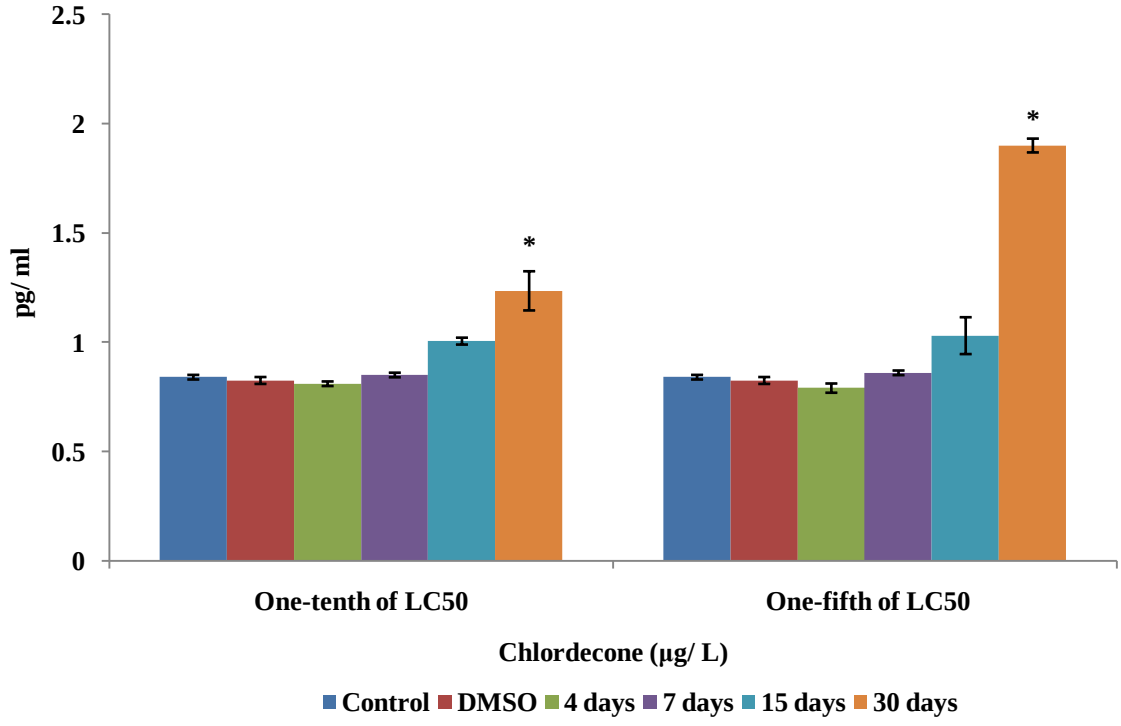


Figure 10: **Effect of chlordecone on the level of estradiol in the female fish, *Pseudetroplus maculatus***



Discussion

5.1 Effects of chlordecone in serum hormone levels in male fish

Measurement of hormones in the circulating blood that are involved in various physiological functions such as energy metabolism, osmoregulation, growth, development and reproduction provide relevant information on the toxic effect of any toxicants in fish. In the present study, male fish when exposed to chlordecone was shown to cause a significant reduction in the level of TSH after 30 days at both sublethal concentrations. Alterations in thyroid hormones are generally correlated with reproductive and developmental impairments. The production and release of thyroid hormones are under the control of TSH, therefore, any changes in TSH lead to structural impairments in testis along with alterations in morphology, motility and concentration of spermatozoa (Kumar *et al.*, 2014). Several other environmental contaminants such as bisphenol A, triclosan, polychlorinated biphenyls and dioxins has been known to cause thyroid disruption in humans and other animals (Seo *et al.*, 1995; Moriyama *et al.*, 2002; Zorrilla *et al.*, 2008).

FSH and LH are released into the blood stream by the stimulatory action of gonadotropin releasing hormone from the hypothalamus. Chlordecone exposure decreased the level of FSH in time-dependant manner only at higher sublethal concentration when compared with the control groups. Decreased production of FSH results in suppressed spermatogenesis, which finally leads to infertility (Simoni *et al*., 1999). In the present study, the decrease in concentration of FSH is correlated with the toxicant-induced alteration in spermatogenesis which is evidenced by decreased motility, viability and concentration of spermatozoa as discussed earlier in chapter 2. The alterations in gonadotropins may be an important reason for the histological response of gonads since the follicular growth is primarily controlled by FSH (Clelland and Peng, 2009). It is evident from the present study that chlordecone exposure decreased the level of LH at higher concentration after 30 days of exposure. The main role of LH is to enable Leydig cells to generate the androgens required for germ cell development in the testis (Simoni *et al.*, 1999). The reduction in the level of LH could lead to changes in the production of testicular sex steroids, especially testosterone which in turn impair the process of spermatogenesis and steroidogenesis leading to infertility in fishes (Schulz *et al.*, 2001). The serum

testosterone concentration was also decreased in duration and concentration-dependant manner in chlordecone exposed groups when compared with the control groups. In the present study, the decreased LH level is responsible for the reduction in serum testosterone level and the result showed similarity to the study on rats after subchronic administration of 17β-estradiol that concomitantly decreased the levels of serum LH and testosterone (Ostby *et al.,* 1999). The decrease in serum testosterone could also be due to the competence action of aromatase enzyme, which converts testosterone to estradiol. Earlier report has shown that glucocorticoid inhibition of FSH also led to the induction of aromatase enzyme in rat granulosa cells (Hsueh and Erickson, 1978).

In the present study chlordecone exposure at two sublethal concentrations increased the level of estradiol in male fish when compared with the control fishes. Thus the estrogenicity of chlordecone in male fish was very well proven by 10-fold to 16-fold increase in the level of estradiol after 30 days of treatment on twofold increase in the concentration of chlordecone. The decrease in the level of testosterone and increase in estradiol concentration in male fish could be due to chlordecone-induced stimulatory activity of aromatase enzyme which converts the testosterone to estradiol (Simpson *et al.,* 1994; Liu *et al*., 2009). The decrease in testosterone concentration could also be due to the decreased activity of 17β-hydroxysteroid dehydrogenase, which has an important role in basal biosynthesis of testosterone (Kwon *et al.,* 2016). Chlordecone also decreased the activities of 3β and17β-hydroxysteroid dehydrogenases in the testis of *P. maculatus* which is discussed in chapter 2.

5.2 Effects of chlordecone in serum hormone levels in female fish

The level of TSH in female fish showed significant decrease only at higher concentration after 30 days of chlordecone exposure. In female fish, chlordecone did not alter the level of FSH while the level of LH decreased significantly at higher concentration after 30 days of exposure. It could be due to pituitary dysfunction in LH release as a result of chlordecone exposure. The decrease in LH may be the cause for the altered histoarchitecture of ovary, which is discussed in chapter 7. The

decrease in LH correlates with the decreased level of serum testosterone after chlordecone exposure at higher concentration after 30 days of treatment. The decreased or unaltered levels of LH and FSH indicate the negative feedback on hypothalamus-pituitary-gonadal axis. Previous literatures have demonstrated that administration of testosterone stimulated an accumulation of LH in the pituitary of juvenile salmonids (Crim and Evans, 1979; 1982). However, castration of spermiating adult coho salmon, *Oncorhynchus kisutch* has been shown to elevate plasma levels of both FSH and LH (Larsen and Swanson, 1997). Gonadal steroids, especially estrogens are known to control the expression of LH in a feedback loop, exerting both positive and negative effects at different stages of reproduction in fish (Eddy and Handy, 2012).

On contrary, chlordecone exposure at both sublethal concentrations significantly increased the level of estradiol after 30 days. The mechanism of chlordecone to induce estradiol may be attributed to the estrogenic properties of chlordecone (Hammond *et al*., 1979). Chlordecone exert the estrogenic activity by binding to ERα and ERβ, where it acts agonist with ERα and an antagonist at ERβ (Kuiper *et al.,* 1998; Lemaire *et al.,* 2006). In addition, chlordecone also interact with nuclear ER thereby stimulating alternative estrogen signaling pathways or triggering enzymes and receptors involved in steroid biosynthesis (Thomas and Dong, 2006; Ray *et al*., 2007; Lee *et al.,* 2008). Similar results were reported, when *Clarias gariepinus* exposed to nonylphenol in which the levels of TSH, FSH, LH and testosterone were decreased with concomitant induction of estradiol (Sayed *et al*., 2012). Sodium arsenite at concentrations of 4, 5 or 6 mg/kg decreased the levels of LH, FSH and testosterone in dose-dependent manner with massive degeneration of all the germ cells in mature wistar rats (Sarkar *et al*., 2003). Male gold fish also showed suppression of testosterone and induction of estradiol after atrazine exposure in concentration and time-dependent manner (Spano *et al*., 2004). Yang *et al.*, (2017) evaluated the steroidogenic effect of bisphenol F and found that it increases estradiol concentration, decreases free testosterone level along with down regulation of 3β and 17β-hydroxysteroid dehydrogenases. The testosterone/estradiol ratio is a sensitive biomarker of abnormal steroid hormone secretion in fish and any change in

the balance between testosterone and estradiol can affect sexual development, reproduction, gametogenesis, and sex determination (Shang *et al*., 2006.) Thus, the variations in different hormones of HPG axis in fish indicate the estrogenicity of chlordecone and its impact on steroidogenesis. As well as, all hormonal parameters proved as the most sensitive tool in monitoring the sublethal toxicity of chlordecone in fish.

The major conclusion drawn from the present study is that the chlordecone induced hormonal changes in the fish, *Pseudetroplus maculatus*. The estrogenic effect of chlordecone was documented in male fish by the increase in the level of estradiol. Imbalance in the male hormones could be the reason for the male reproductive abnormalities such as decrease in sperm concentration, sperm count, viability and motility. The increase in the vitellogenin production in male fish could be also due the increase in the level of estradiol. Thus the environmental signal is known to transmit through hypothalamo-pituitary-gonadal axis that influenced changes in the circulatory level of hormones. Similarly chlordecone exposure imbalance hormonal parameters in female fish, which could ultimately lead to reduced fertility, egg production and reduced gonad size and morphology. Therefore, the hormonal endpoints discussed so far give evidence that chlordecone affect reproductive potential of the exposed fish and this could be one of the reasons for the decline in fish population in the natural ecosystem.

Conclusions

1. Chlordecone exposure disrupts the levels of serum hormones such as TSH, FSH, LH, testosterone and estradiol in *Pseudetroplus maculatus.*

2. Change in the level of TSH reflects alteration in the metabolism of fish, modification in FSH and LH suggest inhibition of gametogenesis, steroidogenesis and vitellogenesis, and alteration in testosterone and estradiol reflects the serious consequence of chlordecone in reproduction of the fish.

3. The results suggest that the endocrine response of fish to chlordecone is mediated through hypothalamo-hypophyseal-gonadal axis.

4. The male reproductive dysfunction is illustrated by increase in the serum level of estradiol, and further estrogenicity of chlordecone is evident in male fish than the female.

5. Hormonal endpoints are the most sensitive parameters to identify estrogenic effects of chlordecone especially at sublethal concentrations.

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CHAPTER 4

Effects of chlordecone on antioxidant status in reproductive tissues

Introduction

1.1 Oxidative stress as biomarkers

The mechanism of free radical generation and defensive system in biological system to eradicate the generated pro-oxidants by the activities of antioxidant enzymes is the recent and rapidly growing field of research. Free radicals are atoms, molecules or ions with unpaired electrons that are highly reactive and take part in several chemical reactions (Halliwell and Gutteridge, 1989). Reactive oxygen species (ROS) includes molecular and singlet oxygen, superoxide anion, hydroxyl radical, hydrogen peroxide etc. In all living organisms, the antioxidant defense system is crucial for protecting the cells from ROS and free radicals produced as a result of aerobic respiration. All cells are continuously exposed to large numbers of oxidants derived from various endogenous and exogenous sources and thus possess basal concentration of these reactive intermediates, which are essential for several processes such as intracellular cell signaling, apoptosis, inflammatory reactions and resistance against micro-organisms (Halliwell and Gutteridge, 1999). However, increase in the amount of reactive oxygen species in biological system is harmful to cells and organisms. Several environmental contaminants are known to induce the production of reactive oxygen metabolites and are known to cause several pathophysiological abnormalities in organisms (Lykkesfeldt, 2007; Lushchak, 2011). The ROS production is counterbalanced by the antioxidant system, but failure of the system results in a condition called oxidative stress, which is the imbalance of pro-oxidant and antioxidant system. The evaluation of oxidative stress is widely used as a biomarker in ecotoxicological studies, which is an effective method to detect health quality assessment of the aquatic ecosystem.

1.2 Sources of reactive oxygen species

All cells are exposed to different types of reactive oxygen species both from endogenous and exogenous sources. The mitochondrion acts as the major organelle responsible for endogenous ROS production and other aerobic events throughout the cell under normal conditions. The rate of free radical production is known to increase in aging cell thereby altering the function of mitochondrion and damaging the membrane integrity (Murphy, 2009; Brunk and Terman, 2002). Several

metabolic enzymes constitute another important endogenous source of ROS, where ROS is produced as a by-product of their activity (Santos *et al*., 2009). Immune cells including white blood cells induce production of ROS during combat with microorganisms. Moreover, numerous pathologies and disease states including cancer as well as impaired metabolism of metals also contribute as important sources of endogenous ROS (Kohen and Nyska, 2002). Exogenous sources of ROS include food, drugs, xenobiotics, pollutants, ultrasound, pathogens, gamma and UV irradiations producing tremendous amounts of different reactive molecules in organisms (Kohen and Nyska, 2002). These exogenous agents such as any xenobiotic pollutants through redox cycling generate ROS as its metabolic by-products. Exposure of living organisms to ionizing and non-ionizing irradiation also constitutes a major source of exogenous ROS (Shadyro *et al.*, 2002).

1.3 Formation of reactive oxygen species

Molecular oxygen is reduced into water molecule in mitochondrial electron-transport chain through four-electron mechanism. Reactive oxygen species are produced from molecular oxygen as a result of the partial reduction of oxygen. ROS are, therefore, generated continuously and are eliminated simultaneously from the biological system. Thus the concentration of ROS is a dynamic parameter, which means the amount of ROS produced is equal to the concentration eliminated (Halliwell and Gutteridge, 1989). Generally 90% of oxygen consumed by an organism is reduced to molecular oxygen through mitochondrial electron transport chain whereas remaining 10% undergo partial reduction through one-electron scheme resulting in ROS formation (Demin *et al*., 1998). The second important source of ROS is by redox steps in electron-transport chain of endoplasmic reticulum that includes catabolism of cellular and foreign chemicals by cytochrome P450 (Malhotra and Kaufman, 2007). Some amount of ROS is also produced in cytosol and peroxisomes by different oxidases such as tryptophan dioxygenase, xanthine oxidase, cytochrome P450 reductase and oxidases of amino acids and glucose (Lushchak, 2011). Apart from the natural sources of ROS production, exposure to environmental pollutants such as metals, aromatic hydrocarbons,

pesticides, nanoparticles, polychlorinated biphenyls, biomaterials, dioxins, phthalate plasticizers etc are known to produced ROS (Bindhumol *et al*., 2003; Chitra *et al*., 2003; Livingstone, 2003). Important reactive oxygen species present in biological systems comprises superoxide anions ($O_2^{-\bullet}$), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2).

1.3.1 Superoxide anions

Superoxide anions are mainly produced in mitochondria by the addition of one electron to the molecular oxygen mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or xanthine oxidase or by mitochondrial electron transport system (Birben *et al.,* 2012). When compared to other free radicals, superoxide anions are relatively stable with low reaction rates within biological components. Superoxide anions inactivate the iron-sulfur cluster containing enzymes of metabolic pathways, thereby releasing free iron in the cell, which leads to the generation of highly reactive hydroxyl radical by Fenton reaction. In addition, the free radicals react with carbonyl compounds and halogenated carbons to produce toxic peroxy radicals as well as initiate the formation of lipid peroxidation in polyunsaturated fatty acids to cause cellular damages (Pryor, 2012). In aqueous solutions, superoxide anions disappear rapidly because of the dismutation reaction by which hydrogen peroxide and oxygen are formed.

1.3.2 Hydroxyl radical

Hydroxyl radical is considered as the most reactive ROS, produced mainly from superoxide anions and hydrogen peroxide in biological systems, which is explained by two reactions namely Haber-Weiss and Fenton reactions. The reactions generate hydroxyl radicals from superoxide anion and hydrogen peroxide, which is catalysed by iron so also referred as iron-catalyzed Haber-Weiss reaction (Kehrer, 2000).

$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$ (1)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ (Fenton reaction) (2)

The net reaction is: $\bullet O_2^- + H_2O_2 \rightarrow O_2 + OH^- + \bullet OH$ ’

The hydroxyl radicals are powerful oxidizing agents with short life span that can react at a high rate with most of the organic and inorganic molecules in the cell, including DNA, proteins, lipids, amino acids, sugars, and metals and initiates peroxidative chain reactions. Hydroxyl radicals are usually generated close to membranes and attacks the fatty acid side chains of the membrane phospholipids to produce lipid hydroperoxide which finally collapse the cell (Halliwell, 1989).

1.3.3 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is a key member of reactive oxygen species next to the superoxide anion and hydroxyl radical which is mainly generated through the respiratory chain cascade and also as byproduct of cellular metabolism (Lennicke *et al.*, 2015). It is produced by xanthine oxidase, amino acid oxidase, and NADPH oxidase within the peroxisomes by using molecular oxygen in metabolic reactions (Granger, 1988; Dupuy *et al*., 1991). H_2O_2 is highly soluble in aqueous solution and can easily penetrate the biological membranes to cause deleterious effects directly or indirectly. H_2O_2 directly elicits oxidative damages to cellular macromolecules and also serves as a source for more deleterious reactive species, such as hydroxyl radicals and hypochlorous acids (Kohen and Nyska, 2002).

Other oxygen-derived free radicals are hypochlorous acid (HClO), peroxyl radicals ($ROO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$). Such free radicals are very transient due to high chemical reactivity that can trigger lipid peroxidation chain reactions by taking an electron from polyunsaturated fatty acids, which disturbs the integrity of cell membranes causing rearrangement of membrane structure (Kohen and Nyska, 2002). Thus lipid peroxidation is a natural metabolic process under normal aerobic conditions and it is one of the most investigated consequences of ROS action on membrane structure and function. Once when the lipid bilayer membrane undergoes peroxidation it becomes rigid and selectively permeable without integrity. Water soluble lipid peroxidation products such as aldehydes have been shown to diffuse into sub-cellular compartments, where it crosslinks with protein aggregates and form DNA adducts giving rise to mutations and altered patterns of gene expression (Davies, 2000).

1.4 Antioxidant defense system

The principal defensive system against the induction of ROS is cellular antioxidant defense system. Antioxidants are chemical substances that contain monohydroxy or polyhydroxy phenol and its function is not to eliminate the oxidants completely from the cell, but it helps to protect against the free radical induced cell damage by slowing down the lipid peroxidation (German, 1999). The action of antioxidants in cells is by slowing down the oxidation so that the free radicals are not produced in secondary, which prevent the cellular damage induced by ROS generation. Based on the mode of action, antioxidants are basically classified as primary, secondary and tertiary antioxidants. Primary antioxidants usually slow down the formation of oxidants. Secondary antioxidants are usually the scavengers of ROS, which effectively involved in removing the free radicals to make it in optimum level. Tertiary antioxidants are involved to repair the oxidized molecules by either dietary or consecutive antioxidants (Singh *et al*., 2003). The action of antioxidants to scavenge ROS is mainly by breaking the side chain of radical that could make it less reactive and less stable or may act as chelator to remove toxic singlet oxygen (Vertuani *et al*., 2004). Based on the defensive mechanisms to protect against the reactive oxygen species, antioxidants are further classified into non-enzymatic and enzymatic antioxidants.

1.4.1 Non-enzymatic antioxidants

Non-enzymatic antioxidants consist of low-molecular-weight compounds, such as water soluble ascorbic acid (vitamin C) and reduced glutathione (GSH) as well as lipid soluble carotenoids, retinol (vitamin A) and tocopherol (vitamin E) (Lushchak, 2011). Vitamin C or ascorbic acid is considered as the most potent antioxidant because it can supply an electron to a species with an unpaired electron, so that it helps to stabilize the radical. The water-soluble vitamin C can directly react with aqueous peroxyl radicals and indirectly by restoring the antioxidant properties of fat-soluble vitamin E (tocopherol) and thus control lipid peroxidation of cellular membranes (Bendich *et al*., 1986). Carotenoids, a group of nearly 600 compounds and the fat-soluble vitamin A possessing structural similarities are known to have

antioxidant properties. The action of antioxidant activity is by the hydrophobic chain of polyene units which can quench singlet oxygen and neutralize the radicals thereby combine and stabilize with peroxyl radicals (Palace *et al*., 1999). Vitamin E is also a powerful lipid soluble antioxidant that predominantly acts as a scavenger of peroxyl radical, disabling the production of harmful effects of free radicals in tissues. By reacting with radical it form tocopheryl radical, which will then be reduced by a hydrogen donor, such as vitamin C and maintain the integrity of long-chain polyunsaturated fatty acids in the membranes of cells (Meagher *et al*., 2001). Thus vitamin E usually functions as free radical scavengers in the cell environment.

Glutathione is the most abundant aqueous antioxidant in all cell compartments which serves as a cofactor for antioxidant enzymes such as glutathione-dependent peroxidases or glutathione-S-transferases (Larsson *et al*., 1983). It has the ability to minimize oxidative stress and downstream the adverse effects associated with oxidative stress. Glutathione mainly minimize the lipid peroxidation of cellular membranes and targets the cells that induces oxidative stress (Parkinson and Ogilvie, 2008). N-acetyl-cysteine, a byproduct of glutathione, is also a popular antioxidant and due to its cysteine residues popularly plays an important role in glutathione maintenance and metabolism. Apart from antioxidant defense function, there are several roles of glutathione in cell compartments, which includes detoxification of electrophilic xenobiotics, modulation of redox-regulated signal transduction, regulation of cell proliferation, storage and transport of cysteine, synthesis of deoxyribonucleotide, regulation of immune responses and leukotriene and prostaglandin metabolism (Sen, 1999). Thus both ascorbate and glutathione play important role to react with hydrogen peroxide and oxygen free radicals.

The destruction of reactive oxygen metabolites have been performed by the functions of both glutathione and ascorbate. However the functions performed by glutathione in free radical scavenging is unique and are not similar to those functions carried out by ascorbate. Glutathione also protects the cells against programmed cell death through the mechanism of proapoptotic and antiapoptotic signaling pathways by regulating and activation of several transcription factors, such

as activator protein-1 (AP-1), nuclear factor-κB cells (NF-κB), and specificity protein-1 (Sp-1) (Masella *et al*., 2005).

1.4.2 Enzymatic antioxidants

All aerobic eukaryotic organisms are equipped with powerful antioxidant enzymes for the eradication of reactive oxygen species. It includes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase (Sies, 1997). Superoxide dismutase (SOD) catalyses the dismutation of superoxide anions into either molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). In mammals, superoxide dismutase exist in three isoforms as cytoplasmic Cu/ZnSOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular Cu/ZnSOD (SOD3), which requires copper or manganese as catalytic metals for the activation (Abreu and Cabelli, 2010).

Cu^{2+}-SOD + $O_2^{-\bullet}$ → Cu^{+}-SOD + O_2

Cu^{+}-SOD + $O_2^{-\bullet}$ + $2H^{+}$ → Cu^{2+}-SOD + H_2O_2

Catalase (CAT) is a ubiquitous extraordinary enzyme found in plant and animal cells. Catalase actively involves in the conversion of powerful and potentially harmful oxidizing agent, hydrogen peroxide, into water and molecular oxygen. One molecule of catalase has been shown to decompose millions of hydrogen peroxide molecules into oxygen and water. Catalase is also known to detoxify several potential harmful toxins such as formaldehyde, formic acid, phenol and alcohol in the body by the process of oxidation using hydrogen peroxide (Weydert and Cullen, 2010).

Glutathione peroxidase (GPx) enzymes remove the hydrogen peroxide by converting it into water.

H_2O_2 + Fe(III)-E → H_2O + O=Fe(IV)E(.+)

H_2O_2+ O=Fe(IV)-E(.+) → H_2O + Fe(III)-E + O_2

2GSH + H_2O_2 → GSSG + $2H_2O$

Glutathione peroxidase (GPx) is an enzyme that belongs to the family of tetrameric enzymes possessing unique amino acid selenocysteine within the active sites and low-molecular-weight thiols, such as glutathione, to reduce hydrogen peroxide and lipid peroxides to the corresponding alcohols. About four types of

glutathione peroxidases encoded by different genes include cellular, gastrointestinal, extracellular and membrane-bound phospholipid hydroperoxide. Cellular glutathione peroxidase (GPx-1) is ubiquitous that help to reduce hydrogen peroxide and fatty acid peroxides, but do not reduce the esterified peroxyl lipids (Arthur, 2000). However, the reduction of esterified lipids occurs by the membrane-bound glutathione peroxidase (GPx-4), which uses different low-molecular-weight thiols as reducing equivalents. Gastrointestinal glutathione peroxidase (GPx-2) localized in gastrointestinal epithelial cells functions to reduce dietary peroxides (Chu *et al*., 1993). Extracellular glutathione peroxidase (GPx-3) is the only member of the peroxidase family that is restricted in the extracellular compartment and it is one of the most important extracellular antioxidant enzymes in mammals (Comhair *et al*., 2001).

The enzyme glutathione reductase (GR) catalyzes the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH), thereby helping to maintain redox status in the cell (Di Giulio *et al*., 1989). Glutathione-S-transferase (GST) is another antioxidant enzyme family that belongs to phase II reaction of xenobiotic biotransformation, which inactivate secondary metabolites, such as unsaturated aldehydes, epoxides, and hydroperoxides (Birben *et al*., 2012). Three major families of GST namely cytosolic, mitochondrial and membrane-associated microsomal GST play important role in the metabolism of eicosanoids and glutathione (Jakobsson *et al*., 1999). Thus the antioxidant system is a complicated parameter which depends on the interaction of both enzymatic and non-enzymatic antioxidants. Measurement of the antioxidant enzyme activities gives ideas about the redox status of the tissues. Imbalance in the pro-oxidant and antioxidant balance in the tissues results in oxidative stress, which is one of the adaptive mechanisms of the animal to escape from wide range of environmental toxicants (Bayir, 2005). Fishes are vulnerable to the attack of ROS and have well-developed antioxidant defense system (Rudneva, 1997). The present chapter discuss on the effects of chlordecone, an environmental toxicant, that generate ROS and cause oxidative damage in various tissues, which is considered as one of the biomarkers to address environmentally induced oxidative stress in the fish, *Pseudetroplus maculatus*.

Review of Literature

Fishes are highly sensitive to environmental pollution and are considered to be the most significant bio-indicators of aquatic pollution. Due to feeding and living in the aquatic environments, fishes are primarily vulnerable and heavily exposed to pollutants because they cannot escape from the deleterious effects of pollutants (Austin, 1998). In order to survive in the contaminated environment, most of the aquatic organisms possess variety of endogenous defensive mechanisms within the body like detoxification, antioxidant protection, excretion and stress responses (Franco *et al*., 2006). Several biomarkers have been developed to assess the toxic impacts of environmental contaminants in different organisms. That is, genetic, biochemical, physiological, behavioral, histological and morphological responses represent useful biomarkers in environmental bio-monitoring (Huggett *et al*., 1992). Oxidative stress and antioxidant parameters are the most commonly used potential biomarkers for evaluating the toxic effects of environmental contaminants. Majority of the studies on oxidative stress in fishes are mainly focused on the effects of environmental contaminants on the activities of antioxidant enzymes and on the potency of lipid peroxidation (Di Giulio *et al*., 1989). Studies have reported that several environmental contaminants such as polynuclear aromatic hydrocarbons, pesticides, polychlorinated biphenyls, organic hydroperoxides, halogenated hydrocarbons, redox cycling drugs, glutathione depleting chemicals, ethanol, heavy metals, ozone, nitrogen dioxide and a number of miscellaneous compounds are powerful oxidants and induce cellular damages (Kappus, 1987).

The toxicity of several xenobiotics is linked with the production of free radicals, which in turn cause many pathophysiological conditions (Kelly *et al.*, 1998). It has also been recommended that oxidative stress biomarkers could be employed in environmental monitoring programs (McCarthy and Shugart, 1990). Several biomarkers of oxidative stress induced by pesticides and other contaminants from field studies were documented (Eufemia *et al.* 1997; Machala *et al.* 2001; Dorval *et al.* 2005). Generally, biomarkers of oxidative stress can be divided into two types such as biomarkers for factors of antioxidant defences and free radical damage in biological systems. The antioxidant status of an organism is demonstrated by assaying antioxidant enzymes such as superoxide dismutase, catalase, xanthine

oxidase (XOD), glutathione peroxidase, glutathione reductase (GR) and glucose 6-phosphate dehydrogenase. It also involves the assay of non-enzymatic antioxidants such as ascorbic acid, reduced glutathione, carotenoids, retinol and tocopherol (Slaninova *et al*., 2009). Similar to other vertebrates, all these antioxidant enzymes have been detected in most of the fish species (Rudneva, 1997). However, the activities vary among the species based on genetic variability (Di Giulio and Meyer, 2008). Comparative studies have shown that the activities of SOD, CAT and GPx activities in fish are lower than those in mammals and birds (Izokun-Etiobhio *et al.*, 1990; Perez-Campo *et al.*, 1993; Rocha-e-Silva *et al.*, 2004). Several intrinsic and extrinsic factors such as age, phylogenetic position, metabolic and reproductive status of fish as well as environmental conditions like food availability and nutritional status, oxygen level, temperature of water, salinity and photoperiod, seasonal variations, toxins present in the water or diseases can either strengthen or decline antioxidant defences (Martinez-A lvarez *et al.*, 2005; Melegaria *et al*. 2013). An increase or decrease in the activities of antioxidant enzymes depends on the intensity and the exposure duration of the stressor as well as the susceptibility of the exposed fish species (Oruc and Usta, 2007). Moreover, the variation in the activities in different organ systems is due to the susceptibility of the organs towards intoxication and occurrence of xenobiotics at different concentrations in the systems due to the differences in the tissue blood volume of fish (Isik and Celik, 2008).

When ROS are not successfully and securely removed, oxidative stress may impair health status of organisms both directly and indirectly. Direct effects include peroxidative damage to chief macromolecules and lipids. Indirectly, the alterations induced by ROS in cellular membranes and components can modify metabolic pathways, resulting in altered physiology and several pathological conditions (Miller *et al.*, 1993). Initially, the reactive oxygen metabolites attacks and oxidize lipids, proteins, polysaccharides, DNA, and other macromolecules. Then these oxidized molecules abstract electrons from other molecules, resulting in a chain reaction along with the generation of lipid peroxides. This reaction, if not controlled, can cause widespread tissue damage, which may affect membrane permeability, enzyme function and expression of genes (Miller *et al.*, 1993). Thus, the most commonly

used biomarkers of free radical damage in biological system are the products of lipid peroxidation as aldehydes, especially malondialdehyde (MDA), ketones and the determination of diene conjugation from the polyunsaturated fatty acids (PUFA) (Valavanidis *et al*., 2006). Malondialdehyde is the major secondary byproduct of peroxidation of polyunsaturated fatty acids. The biomarkers of *in vivo* oxidative damage to DNA are products of the specific modifications and hydroxylations of purine and pyrimidine bases, products of damage to the deoxyribose phosphate backbone and protein-DNA cross-links (Valavanidis *et al.*, 2006). The protein oxidation contributes another type of damage to biological systems by free radicals. Oxidative damage mainly focus on the estimation of carbonyl derivates of proteins, particularly the oxidation products of tyrosine (Huggins *et al.* 1993) and phenylalanine (Valavanidis *et al.*, 2006). Detection of γ- glutamyl semialdehyde and 2-amino-adipic semialdehyde is another evidence of protein oxidative damage (Valavanidis *et al.*, 2006).

Lipid peroxidation and DNA damage contribute important toxic responses of free radical mediated reactions in mammalian and piscine systems. Lipid peroxidation has been used as a measure of xenobiotic-induced oxidative stress. For example, endosulfan has been shown to induce lipid peroxidation in the testes of rat (Chitra *et al*., 1999). Bisphenol A has been shown to induce lipid peroxidation in epididymal sperm (Chitra *et al*., 2003) and liver of rats (Bindhumol *et al*., 2003) as well as lindane has been shown to induce lipid peroxidation in rat testes (Sujatha *et al.*, 2001. In a study of *Channa punctatus*, endosulfan exposure resulted in induction of lipid peroxidation in liver, kidney and gill tissues (Pandey *et al.*, 2001). Similarly, glyphosate has been shown to cause an increase lipid peroxidation in liver and muscle tissues of silver catfish (Glusczak *et al.*, 2007).

A wide variety of different xenobiotics have been shown to induce oxidative stress in the gonads in concert with the suppression of antioxidant mechanisms. At the level of the testes, oxidative stress is capable of disrupting the capacity of germinal epithelium to differentiate normal spermatozoa as well as steroidogenic capacity of Leydig cells (Naughton *et al*., 2001; Hales *et al*., 2005). Spermatozoa are highly susceptible to damages caused by ROS because the plasma membranes of

spermatozoa have high levels of polyunsaturated fatty acids (Alvarez and Storey 1995). This lead to increased production of ROS which in turn could result in impaired sperm functions (Alvarez *et al*., 1987). Spermatozoa contain relatively low levels of antioxidant enzymes (Agarwal *et al.*, 2003) because most of the genes for antioxidant enzymes are transcriptionally silent (Aitken and Graves 2002), making them less scope for protection against the detrimental effects of ROS. Moreover, spermatozoa produce more ATP to facilitate the motility, and this increased energy metabolism contributes a major source of ROS (Koppers *et al.,* 2008). At the level of the isolated spermatozoa, ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of the cells and ability to support normal embryonic development (Agarwal *et al.,* 2006; Aitken and Baker, 2006). ROS production in semen has been associated with loss of sperm motility, decreased capacity for sperm–oocyte fusion and loss of fertility (Griveau and Le Lanno, 1997). Thus oxidative stress is clearly a dominant feature in the etiology of male infertility.

ROS also impairs the functioning of steroidogenic enzymes by diminishing the supply of NADPH and also by NADPH-supported lipid peroxidation (Sies, 1985). Peroxidative inactivation of steroidogenic enzymes can limit synthesis of steroid hormones under oxidative stress which in turn affect the reproduction (Takayanagi *et al.,* 1986). ROS also affects the functioning of hydroxylases specific to cytochrome P-450 in the adrenal microsomes which in turn inhibit the synthesis of estrogens and androgens (Yanase *et al.,* 1991). Several industrial and environmental toxicants are known to impair male fertility by inducing the state of oxidative stress in the testes. Vanadate is one among them which induces lipid peroxidation in the testes along with significant inhibition of testicular SOD and catalase with the disruption of 3β- and 17β-hydroxysteroid dehydrogenase activities (Chandra *et al.,* 2007). In rats, exposure to cyclophosphamide has been shown to reduce the intratesticular concentration of testosterone and slows down the expression of antioxidant enzymes such as SOD, CAT and GPx along with inhibition of steroidogenic enzymes thereby causing impaired spermatogenesis (Ghosh *et al.,* 2002). Likewise, administration of estradiol showed decreased activities of antioxidant enzymes, disruption of spermatogenesis, and increase in

germ cell apoptosis along with concomitant induction of lipid peroxidation in rats (Chaki *et al*., 2006). In addition, several other environmental contaminants such as methoxychlor, bisphenol A, nonylphenol, lindane, endosulfan and quinalphos are also capable of impairing male fertility by inducing testicular oxidative stress (Debnath and Mandal, 2000; Chitra *et al.,* 2002; Latchoumycandane and Mathur, 2002; Chitra *et al.,* 2003; Kabuto *et al.,* 2003).

ROS production plays several physiological and pathological roles in the female reproductive tract such as oocyte maturation, ovarian steroidogenesis, corpus luteal function and luteolysis (Behrman *et al*., 2001). Lower concentrations of ROS has been shown to damage ovarian development by negatively affecting steroid hormone biosynthesis, probably through the oxidative modification of lipids, proteins, carbohydrates, and DNA in rats (Margolin *et al.*, 1990). Wild white sucker, *Catostomus commersoni* collected from environments receiving pulp and paper mill effluent discharges in northern Ontario showed gonadal oxidative stress, decreased gonadal weight and lowered concentrations of circulating plasma steroids such as estradiol and testosterone (Oakes *et al.,* 2003). Ovaries of *Alburnus tarichi* in Lake Van, Turkey showed altered antioxidant enzymes along with histological impairments in which the oocytes were developmentally blocked and arrested at the previtellogenic stage with atresia and severe fibrosis (Kaptaner, 2015).

Besides, oxidative stress has been related to a broad variety of processes such as mutagenesis, cell transformation, cancer, arteriosclerosis, heart attacks, chronic inflammatory diseases, disorders of the central nervous system, and a wide array of age-related dysfunctions, implicated in the pathophysiology of many diseases and syndromes (Rahman, 2007). Free radicals are found to be concerned with both initiation and promotion of multistage carcinogenesis, DNA damage, activate procarcinogens, and alter the cellular antioxidant defense system (Klaunig *et al.,* 2011). Elevated levels of ROS and down regulation of various antioxidant enzymes and free radical scavengers are associated with different types of cancers (Waris and Ahsan, 2006). In diabetic patients, elevated levels of glucose induce oxidative stress that is eventually reflected by the increased malondialdehyde levels and induction of antioxidant enzymes, which are responsible for diabetic

complications (Ahmed *et al*., 2006). Oxidative stress has been reported to play a major role in the pathogenesis and progression of many liver diseases (Webb and Twedt, 2008)

Many xenobiotics, such as pesticides, may cause oxidative stress leading to the generation of ROS and alterations in antioxidants or free oxygen radicals scavenging enzyme systems in aquatic organisms (Livingstone, 2001). One of the freshwater fish, *Wallago attu* collected from two polluted sites of river Yamuna nearby Agra and Panipat has been shown to alter the activities of antioxidant enzymes along with high degree of lipid peroxidation in gill, liver and kidney (Pandey *et al.*, 2003). Increase in the activity of superoxide dismutase and reduction of catalase has been observed in the liver tissue of fish *Cyprinidae* sampled from a polluted area in Seyhan Dam Lake, Turkey when compared with the reference area (Gul *et al.*, 2004). Freshwater crab, *Sinopotamon henanense*, when exposed to cadmium showed decreased activities of SOD, GPx and CAT with concomitant induction of lipid peroxidation and hydrogen peroxide generation in the testis (Wang, 2013). Reduction in the activities of catalase and glutathione along with increased lipid peroxidation in the liver and ovary of zebrafish has been reported after cadmium chloride exposure (Sunaina and Ansari, 2015). Fluoride exposure significantly elevated the level of lipid peroxidation, CAT, SOD, and GST with decrease in GPx activity in the ovary and liver tissues of *Heteropneustes fossilis* (Yadav *et al.*, 2015). One of the estrogenic contaminants, nonylphenol has been shown to alter the activities of SOD, CAT and GR and induced production of ROS, which is evidenced by the induction of hydrogen peroxide generation and lipid peroxidation in the testes and ovary of *Etroplus maculatus* (Asifa and Chitra, 2016a, b). *Oreochromis niloticus* sampled from Rosetta branch of River Nile showed accumulation of heavy metals such as iron, zinc, copper, manganese and cadmium in liver and muscle tissues of the fish with altered activities of SOD, CAT, GPx, GST, GR and lipid peroxidation (Khalil *et al*., 2017). Thus xenobiotic induced alterations in the levels of antioxidant enzymes and induction of ROS in different tissues, especially in the gonads is responsible for several reproductive impairments in exposed organisms.

Materials and Methods

3.1 Selection of test concentrations and treatment

Based on the LC_{50}–96 h value of chlordecone, two sublethal concentrations - 3.5 µg/ L and 7 µg/ L (1/10th and 1/5th of LC_{50}) were selected and exposed to both male and female fishes for 24, 72 and 96 h durations. Ten animals were maintained in all treatment groups, including positive (DMSO) and negative controls.

3.2 Preparation of samples

At the end of every treatment period, fishes caught by using a small dip net, one at a time with least disturbances were decapitated and gonads were dissected out and stored at 4ºC until the biochemical analyses were performed. A 1% (w/ v) homogenate of testis and ovary were prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 800*g* for 15 min at 4ºC to obtain the supernatant, which was then used for the biochemical analyses. Protein was estimated by the method of Lowry *et al* (1951) with bovine serum albumin (BSA) as the standard and the antioxidant enzymes were assayed by the following methods.

3.2.1 Assay of superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund (1974). The assay mixture contained 2.4 mL of tris hydrochloric acid buffer (50 mM) containing 1 mM EDTA (pH 7.6), 300 µL of pyrogallol (0.2 mM) and 100 µL enzyme source. Increase in the absorbance was measured immediately at 420 nm against enzyme blank for 3 min at 10 sec intervals on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as nmol pyrogallol oxidised/ min/mg protein.

3.2.2 Assay of catalase

Catalase (EC. 1.11.1.6) was assayed by the method of Claiborne (1985). The assay mixture contained 2.4 mL of phosphate buffer (50 mM, pH 7.0), 10 µL of hydrogen peroxide (19 mM) and 50 µL enzyme source. Decrease in absorbance was measured immediately at 240 nm against enzyme blank at 10 sec intervals for 3 min

on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as μmol of hydrogen peroxide consumed/ min/mg protein.

3.2.3 Assay of glutathione reductase

Glutathione reductase (EC. 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1985). The assay mixture contained 1.75 mL of phosphate buffer (100 mM, pH 7.6), 100 μL of NADPH (200 mM), 100 μL of EDTA (10 mM), 50 μL of glutathione oxidized (20 mM) and 50 μL of enzyme source. Disappearance of NADPH was measured immediately at 340 nm against enzyme blank at 10 sec intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as nmol of NADPH oxidised/ min/mg protein.

3.2.4 Estimation of hydrogen peroxide generation

Hydrogen peroxide generation was assayed by the method of Pick and Keisari (1981). The assay mixture contained 1.64 mL phosphate buffer (50 mM, pH 7.6), horseradish peroxidase (8.5 units/ mL), 30 μL of phenol red (0.28 nM), 165 μL of dextrose (5.5 nM) and 600 μL of enzyme source was incubated at 32°C for 30 min. Reaction was terminated by addition of 60 μL of 10N sodium hydroxide. Absorbance was read at 610 nm against enzyme blank on a Shimadzu UV-Visible Spectrophotometer. The quantity of hydrogen peroxide produced was expressed as nmol hydrogen peroxide generated/ mg protein. For the preparation of standard curve, known amount of hydrogen peroxide and all the above reagents except enzyme source were incubated for 30 min at 32°C and then 60 μL of sodium hydroxide (10N) was added and optical density was read at 610 nm.

3.2.5 Estimation of lipid peroxidation

The level of lipid peroxidation was measured by the method of Ohkawa *et al*. (1979). The stock solution contained equal volumes of trichloroacetic acid 15% (w/ v) in 0.25N hydrochloric acid and 2-thiobarbituric acid 0.37% (w/ v). One volume of the test sample and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed and heated for 15 min in a boiling water bath. After

cooling on ice the precipitate was removed by centrifugation at 1000 *g* for 15 min and absorbance was measured at 532 nm against enzyme blank. The values were expressed as μmol of malondialdehyde formed/ mg protein. A standard curve was prepared with the known amount of malondialdehyde and all the above reagents except enzyme source. The optical density was read at 532 nm

3.3 Statistical analyses

All biochemical estimations were repeated twice so as to reduce statistical errors. Statistical analysis was performed using one-way analysis of variance (ANOVA) using statistical package SPSS 19.0. For the comparison of equal variances, Duncan's Multiple Range test was done as post-hoc test. Differences were considered to be significant at $P<0.05$ against the control groups. Data are presented as mean ± SD for ten animals per group.

Results

4.1 Effects of chlordecone in gonadal weights of the fish, *Pseudetroplus maculatus*

Exposure to chlordecone at 3.5µg/ L and 7µg/ L concentrations showed significant ($P<0.05$) reduction in the weight of testis and ovary after 96 h of exposure when compared with the control groups (Figures 1 and 2). Fish exposed to DMSO (positive control) showed no significant changes in the weights of gonads and found similar to negative control group (Figures 1 and 2).

4.2 Effects of chlordecone on the antioxidant status in the testis of the fish

Testicular antioxidant enzymes as superoxide dismutase, catalase and glutathione reductase showed significant ($P<0.05$) decrease in time-dependent and concentration-dependant manner when compared to the control groups (Figures 3-5). Meanwhile, the levels of hydrogen peroxide generation and lipid peroxidation showed significant increase at both sublethal concentrations (Figures 6 and 7).

4.3 Effects of chlordecone on the antioxidant status in the ovary of the fish

In ovary the activity of superoxide dismutase and glutathione reductase decreased significantly ($P<0.05$) at both sublethal concentrations in time-dependent manner (Figures 8 and 10). Catalase activity was decreased significantly only after 96 h at 3.5µg/L concentration whereas a time-dependent significant ($P<0.05$) decrease was observed after 7µg/L concentration of chlordecone (Figure 9). The levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) only after 96 h in one-tenth of LC_{50} concentration, whereas after highest sublethal concentration there was a significant increase after 72 and 96 h of chlordecone treatment in time-dependent manner when compared to the control groups (Figures 11 and 12).

Figure 1

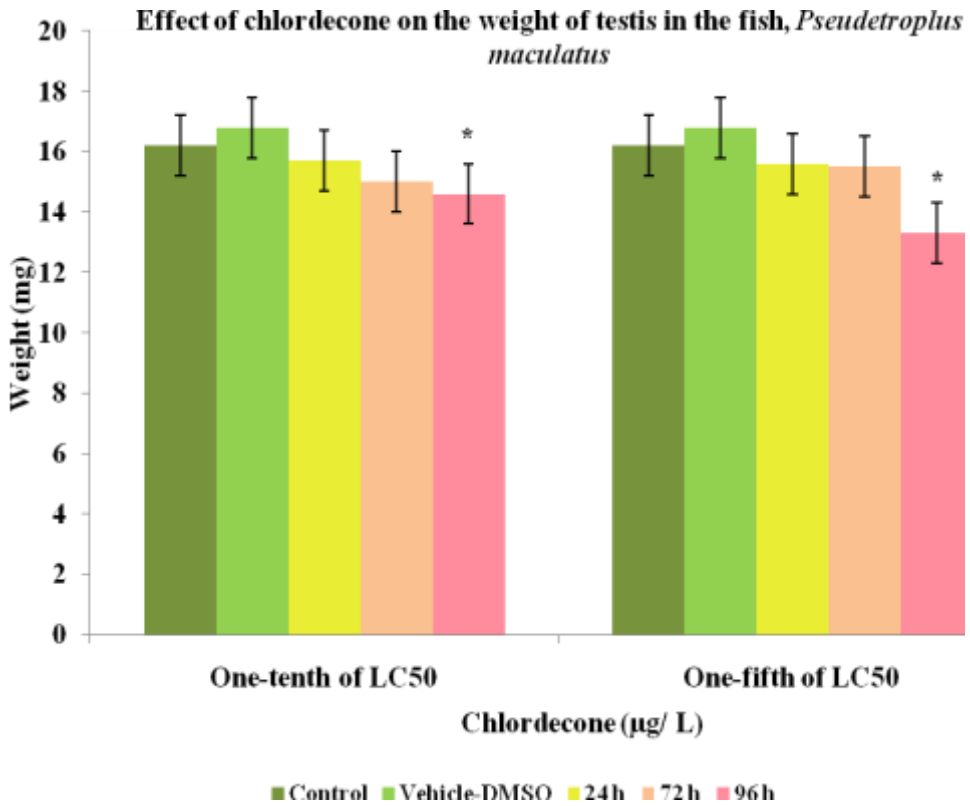


Figure 2

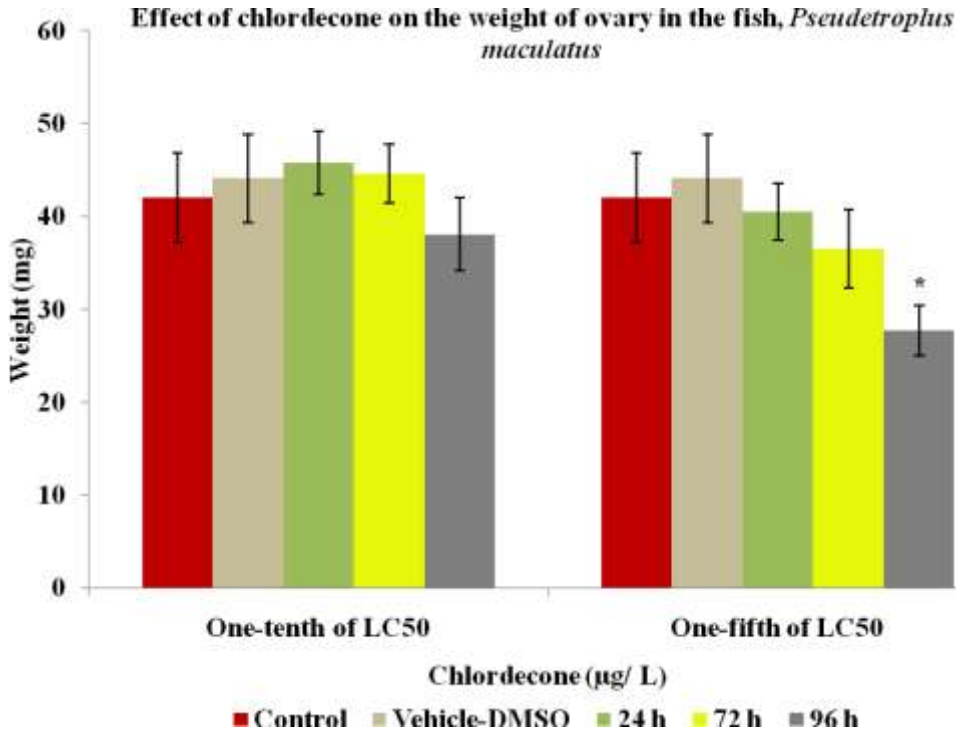


Figure 3

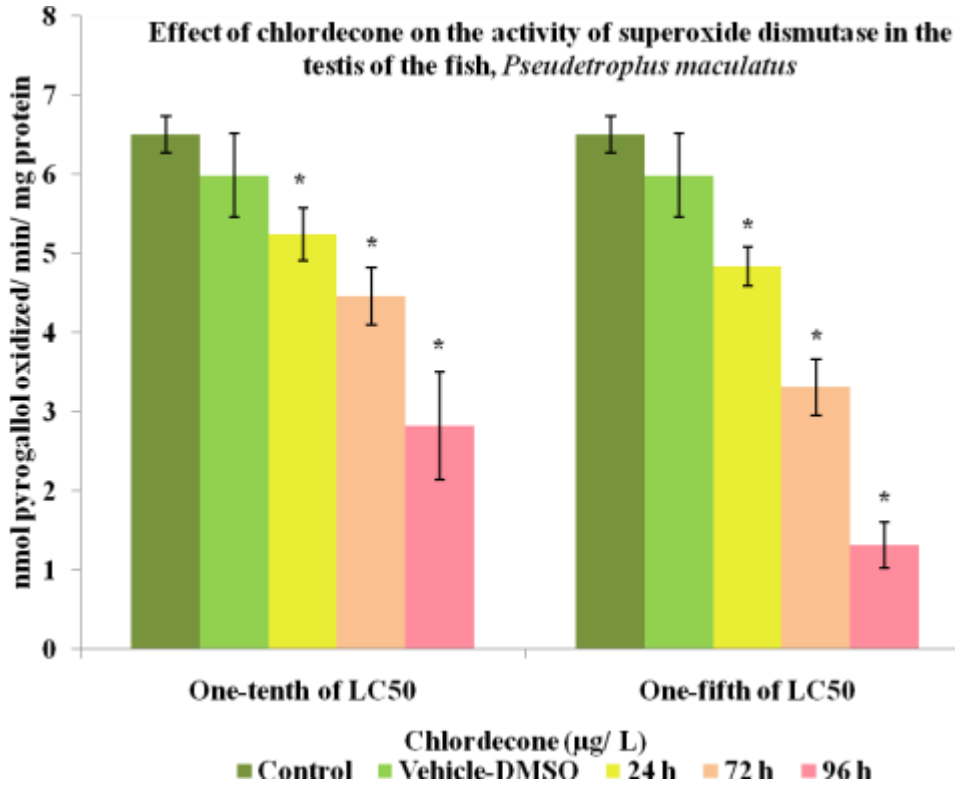


Figure 4

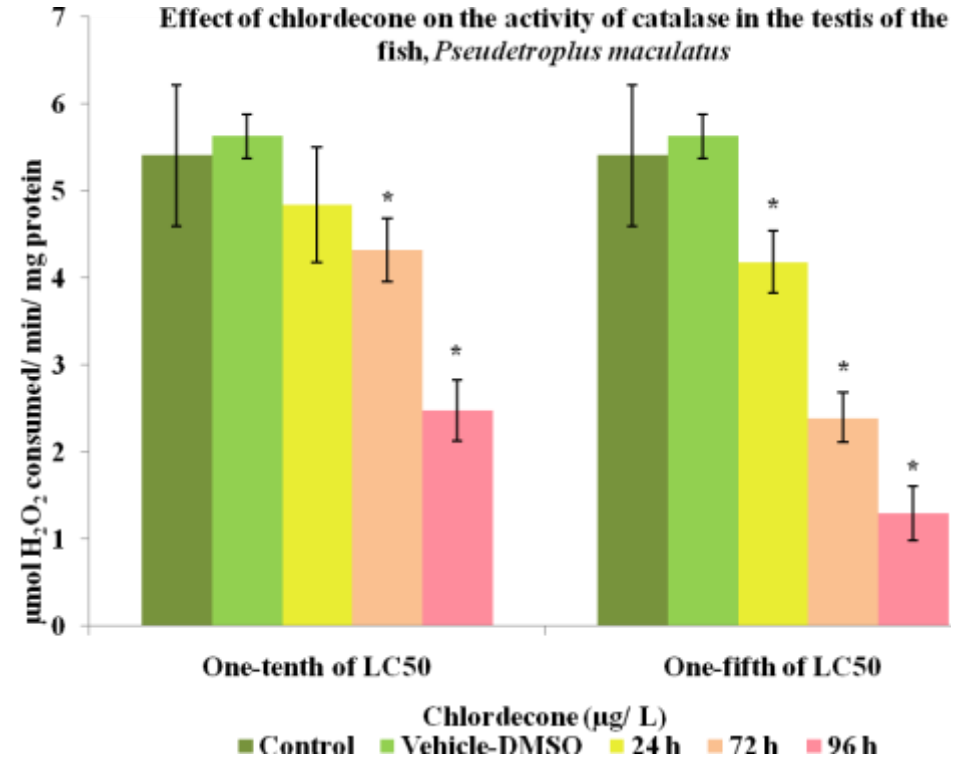


Figure 5

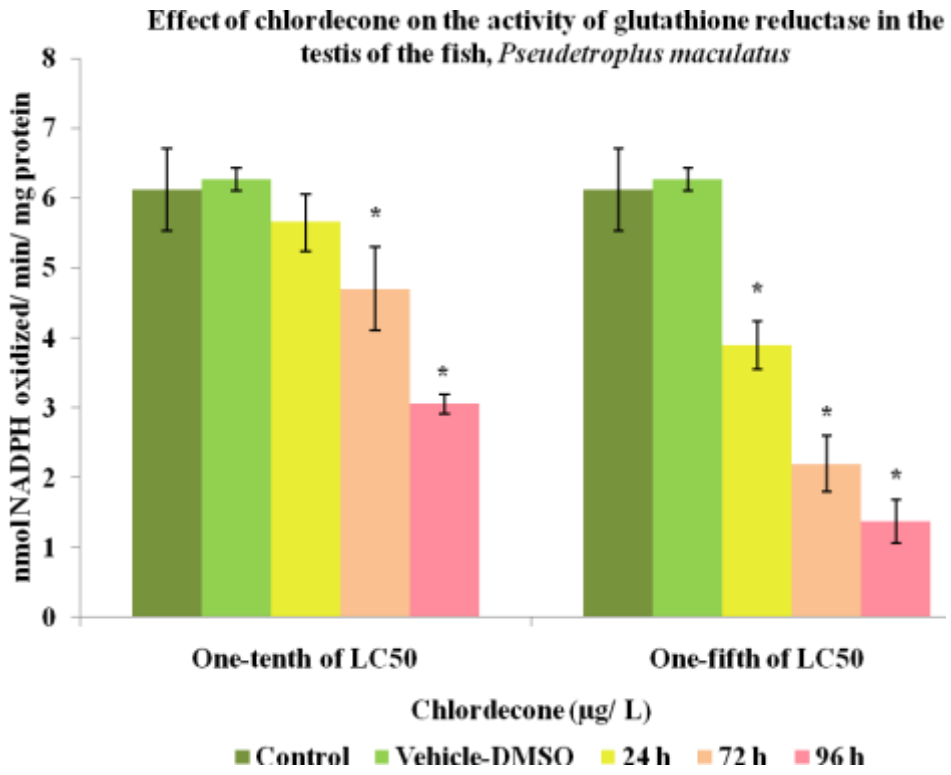


Figure 6

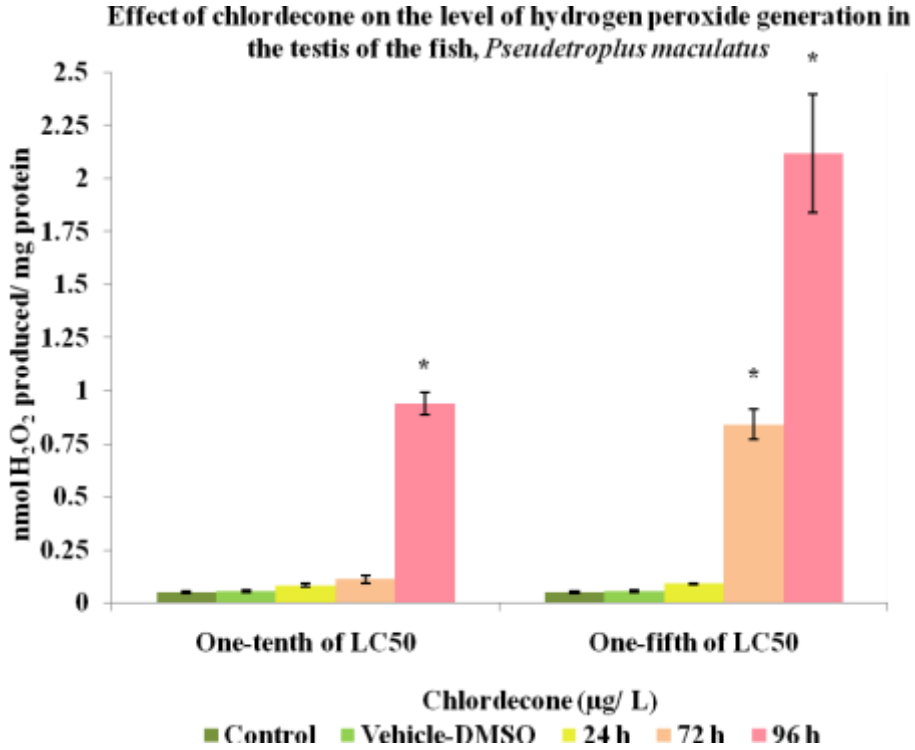


Figure 7

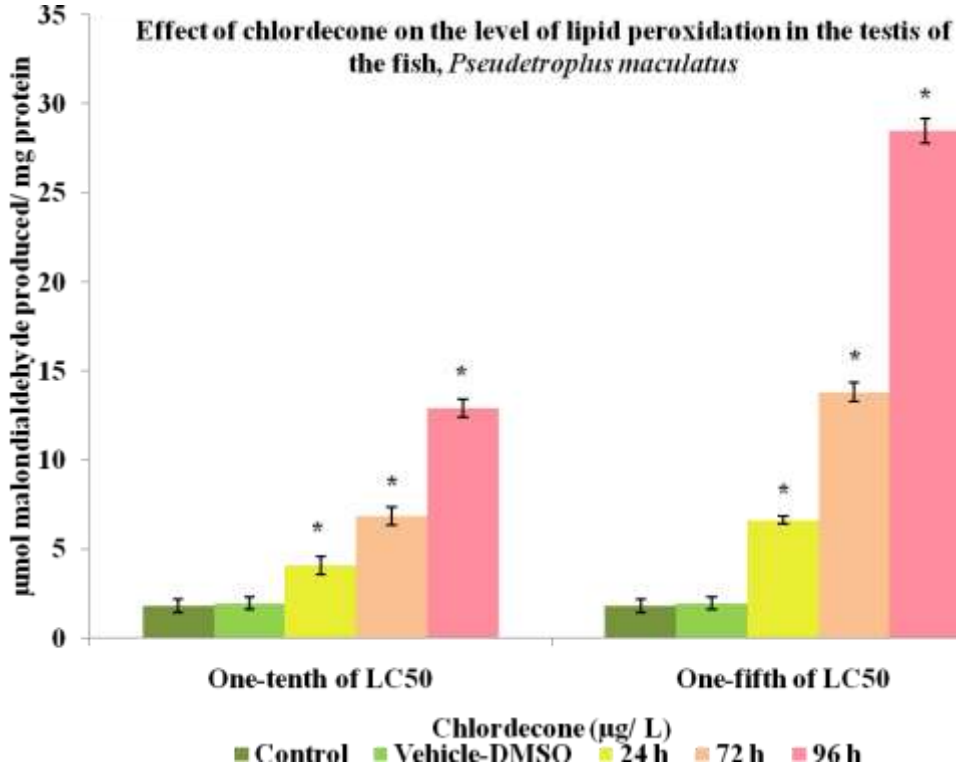


Figure 8

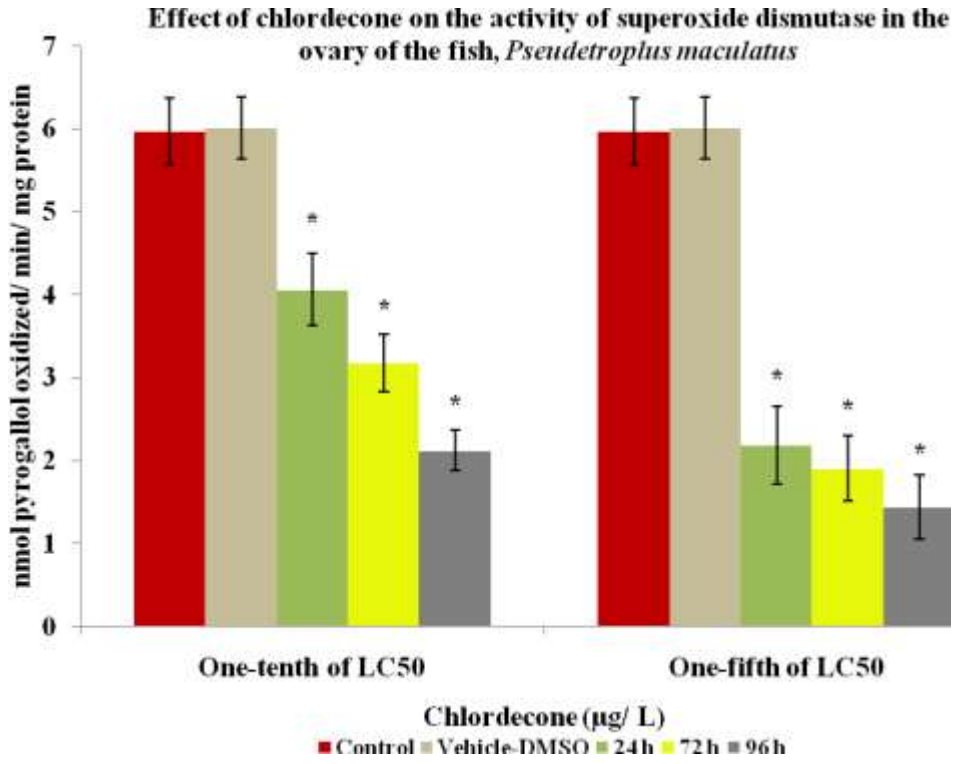


Figure 9

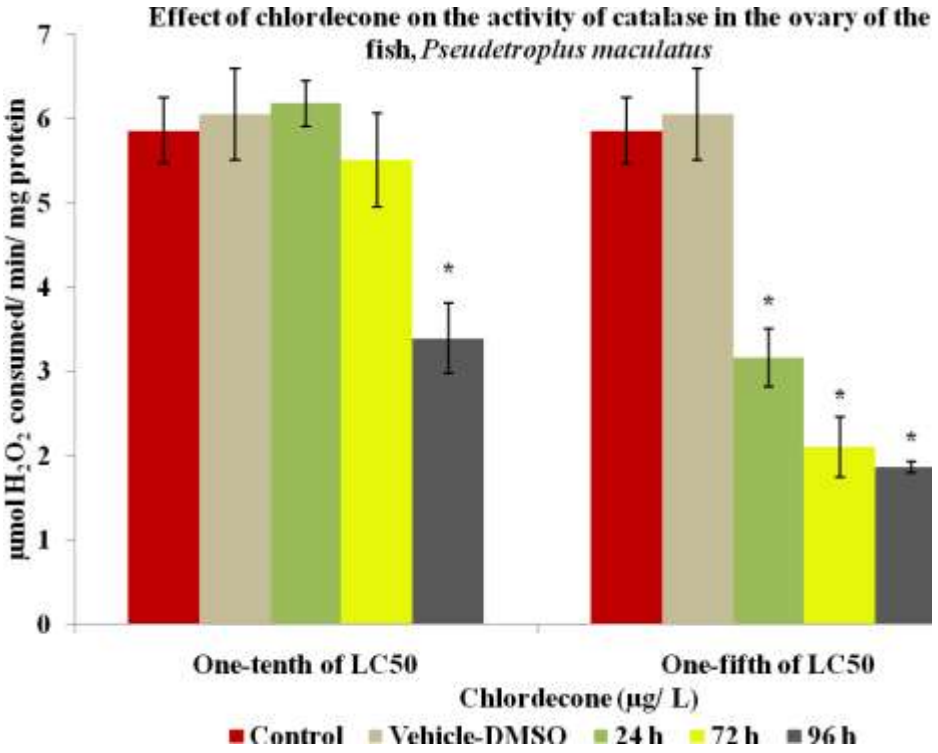


Figure 10

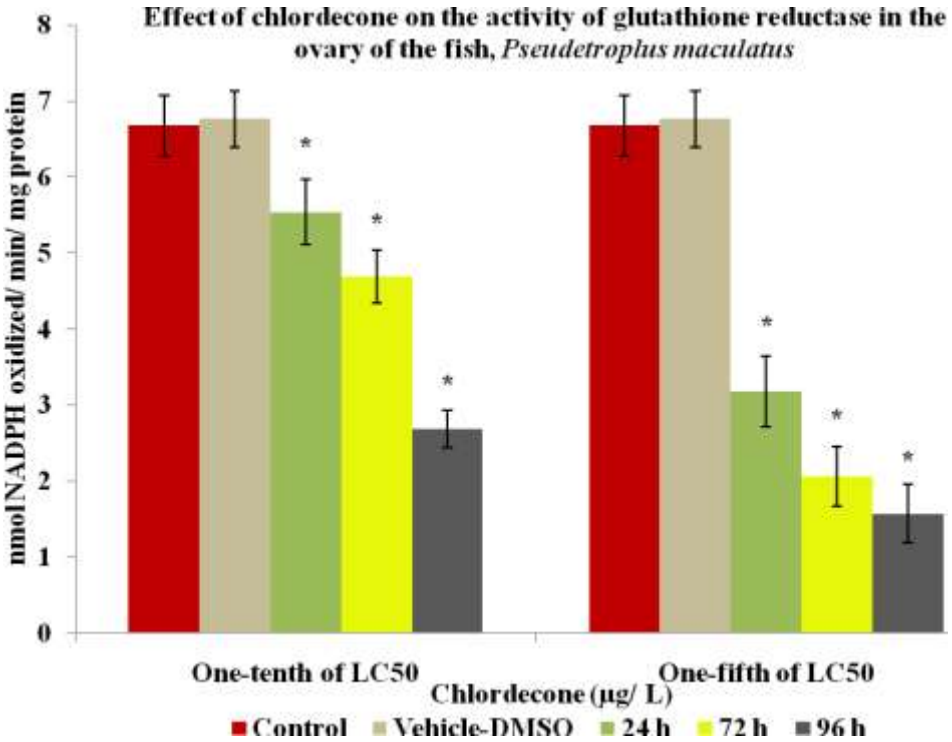


Figure 11

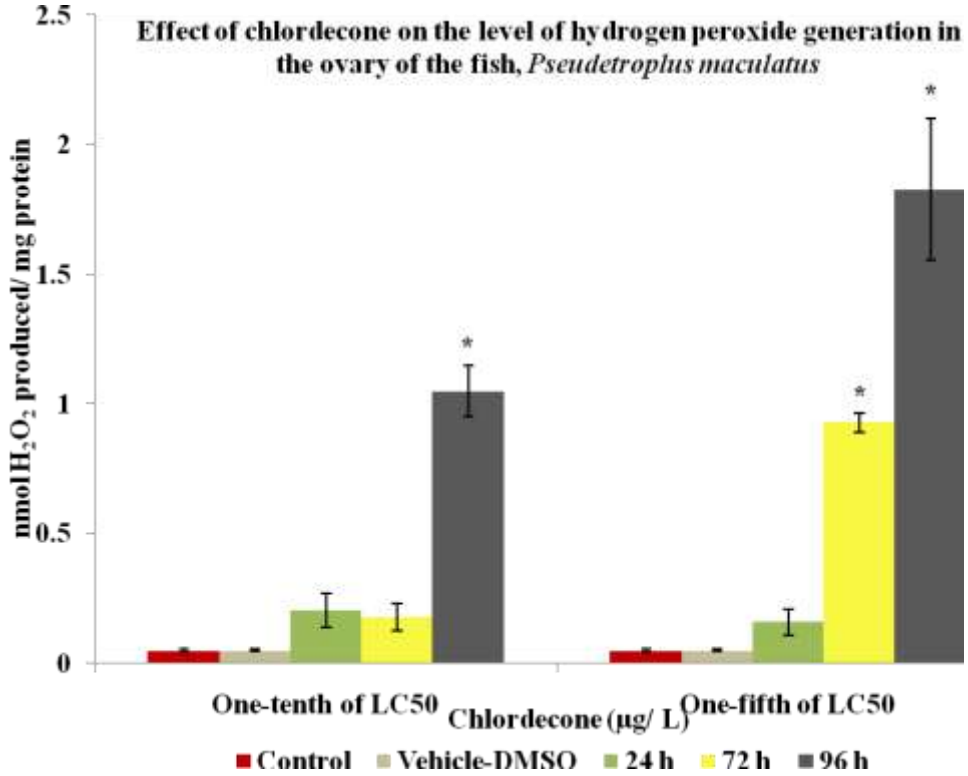
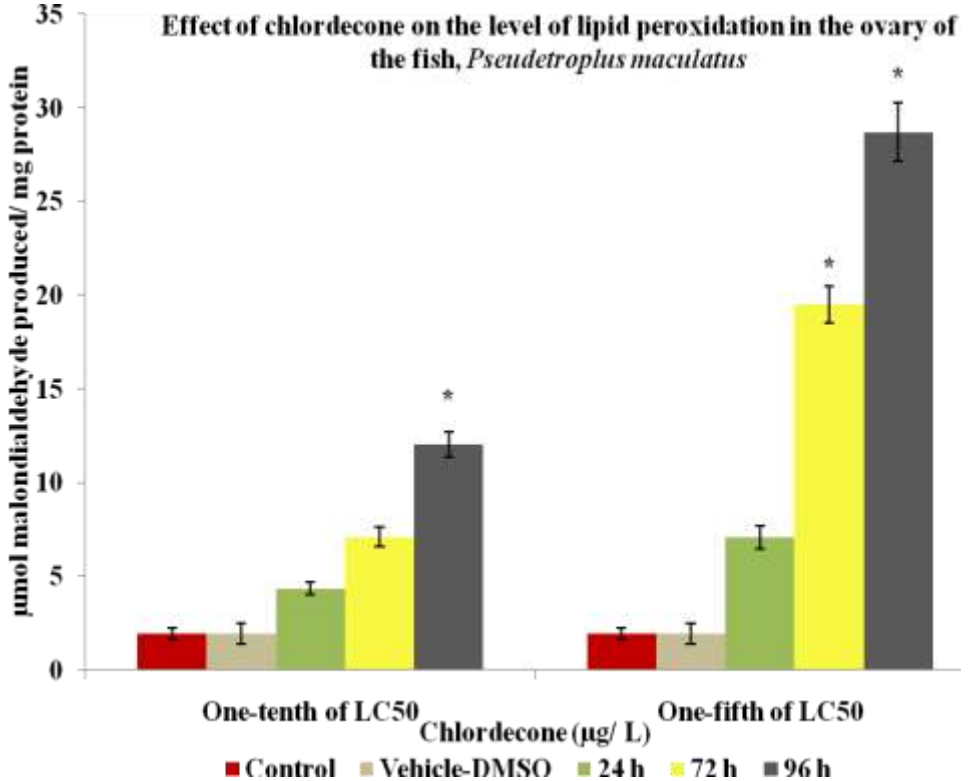


Figure 12



Discussion

5.1 Effects of chlordecone on the weights of testis and ovary of the fish

Contamination of aquatic ecosystems with pesticide is a great concern because it can directly elicit toxicity to species inhabiting in the polluted area and indirectly affect the health status of higher organisms including humans through the food chain. Most of the pesticides are lipophilic compounds and are able to bioaccumulate in the fatty tissues as well as leaches into milk and blood (William *et al.,* 2008). Bioaccumulation of pesticides in the fatty tissues even at low concentrations for prolonged period of time might pose potential adverse effects in the exposed organisms (Metcaff, 1997). In the present study, *Pseudetroplus maculatus* was exposed to chlordecone at one-tenth and one-fifth of LC_{50} concentrations (3.5 and 7µg/L) for 24, 72 and 96h durations. DMSO-treated fish (vehicle) did not showed any noticeable alterations in all parameters tested in the study and the data obtained are similar to those of the solvent-free control group. In toxicology studies, analysis of organ weight is the most susceptible indicator for detection of possible harmful effects of toxicants exposure (Bailey *et al*., 2004). The decrease in gonad weights is an indication of intensity of the toxicant on cellular and tissue damage (Sangha *et al*., 2011). The results of the study have shown that chlordecone treatment decreased the weights of testis and ovary at 96h of exposure and this could be due to toxicant-induced atrophy or necrosis in the tissues, which is evident in the histopathological modifications as described in chapter 7.

5.2 Effects of chlordecone on the antioxidant status in the testis of the fish

Fishes are very important to humans because they contribute important sources of proteins and lipids. Like other aquatic animals, fishes are continuously exposed to wide range of pesticides during the course of life cycle. Pesticides are mostly absorbed through gills, skin or alimentary ducts in fish (Schlenk, 2005; Banaee, 2012) and could significantly cause damage to certain physiological and biochemical processes when they enter into various organs of fishes (Banaee *et al.*, 2011). The toxicity of most of the pesticides are associated with the production of reactive oxygen species (ROS), which result in oxidative damage to cellular structures such as carbohydrates, proteins, lipids and nucleic acids and modify its

functions (Livingstone, 2003). In aquatic ecotoxicology, fish is widely used as beneficial model organisms to study environmental toxicant induced oxidative stress as there are similarities in the antioxidant defense system of fish and mammalian system and therefore, used to evaluate the link between toxicant exposure and oxidative toxicity.

The antioxidant system of fishes is highly sensitive to various environmental contaminants, which is evidenced by the alterations in the activities of antioxidant enzymes along with production of free radicals or ROS. To counteract the toxic effects, the cells or tissues naturally initiates the production of several defensive antioxidant enzymes to prevent from cellular injury and therefore to maintain normal cellular oxidative homeostasis (Halliwell and Gutteridge, 1999). Several biomarkers are used to measure cellular damages and the most intensively used markers are the assay of activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and/or peroxidase system and the level of lipid peroxidation (Yang and Lee, 2015). In recent years the association of ROS production and male reproductive abnormality has become a real concern as it seriously affect the quality of sperm and its functions. Testicular oxidative stress develops as a result of an imbalance between ROS production and its scasvenging activities (Sikka, 2001).

In the present study, chlordecone at two sublethal concentrations (3.5µg/L and 7µg/L) decreased the activities of superoxide dismutase, catalase and glutathione reductase in testis in time-dependent and concentration-dependant manner when compared to the control groups. The present results suggest that the uncontrolled production of free radicals exceeds the antioxidant capacity of testicular cells. This is principally due to the fact that the testicular cell membranes are highly rich in polyunsaturated fatty acids rendering them highly vulnerable to oxidative damage and hence lipid peroxidation (Aitken *et al*., 2010). It was confirmed by the excessive generation in the levels of hydrogen peroxide and lipid peroxidation in testis after chlordecone exposure. Excessive production of lipid peroxidation could lead to disruption of membrane fluidity and loss of sperm functions as sperm motility, viability and sperm count (Sikka, 2001; Agarwal *et al.*,

2014). It was confirmed in the present study that chlordecone exposure disrupts the sperm functional parameters as sperm motility, viability and sperm count and this could be due to the induction of oxidative stress, which was discussed in chapter 2.

5.3 Effects of chlordecone on the antioxidant status in the ovary of the fish

The formation of reactive oxygen species has several physiological and pathological roles in the female reproduction. It is involved in the modulation of physiological reproductive functions as differentiation and maturation of ovary, ovarian steroidogenesis and fertility (Behrman *et al*., 2001). Thus reactive oxygen species are considered as 'double-edged sword' which functions as a prime signaling molecules for several physiological processes and at the same time excess amount of ROS have role in pathological process of female reproduction. Therefore, by measuring the activities of antioxidant enzymes and the production of lipid peroxidation roughly give the assessment of antioxidant status of the ovarian tissue on exposure to one of the environmental contaminants, chlordecone. Exposure to chlordecone showed decrease in the activities of superoxide dismutase and glutathione reductase in concentration and time-dependant manner in the ovary. The activity of catalase enzyme was decreased significantly only at 96 h of chlordecone exposure at 3.5µg/L, whereas at 7µg/L it showed a time-dependant decrease when compared with the control fishes. The observations clearly illustrate that the failure of antioxidant defense system in the reproductive tissues of the fish is to eradicate the ROS production as a result of chlordecone exposure.

It is further evident by the increased levels of hydrogen peroxide generation and lipid peroxidation in the ovary at both sublethal concentrations of chlordecone. Lipid peroxidation is one of the consequences of uncontrolled oxidative stress in cells, in which the oxidants such as free radicals or non-radical species attack lipids in cell membranes leading to cell damage (Marnett, 1999). Chlordecone exposure caused continuous generation of ROS in the ovary of fish that may increase the risk for intrafollicular oxidative damage, poor oocyte maturation, low egg production and eventually leads to female infertility in exposed fish.

The SOD–CAT system is considered as the first line of defense against reactive oxygen toxicity (Pandey *et al.,* 2003), and these enzymes are commonly used as biomarkers, which indicate the production of reactive oxygen species in tissues (Monteiro *et al.,* 2006). In the present study, chlordecone at both sublethal concentrations inhibits the activity of antioxidant enzymes along with induction of hydrogen peroxide generation and lipid peroxidation in both testis and ovary of the fish. The excess production of hydrogen peroxide may reduce SOD activity, while the superoxide anion may be responsible for decreased CAT activity (Bagnyukova *et al.,* 2006). Thus, it is logical to assume that the generation of hydrogen peroxide as a result of chlordecone exposure could be responsible for the reduction observed in the activity of SOD. Similarly, the reduction in CAT activity may be due to superoxide ions, which are probably not being neutralized efficiently by SOD. It was clearly demonstrated that the gonads are unable to neutralize the ROS production owing to chlordecone exposure, which in turn could have reacted with membrane lipids, producing lipid peroxidation and thus leading to oxidative stress (Hermes-Lima, 2004). To brief, there is a strong possibility that induction of oxidative stress in the reproductive tissues, testis and ovary, would lead to failure of reproductive functions that directly affect the ability of reproduction and finally decline in the fish population.

Conclusions

1. Chlordecone exposure at sublethal concentrations induced oxidative stress in the reproductive tissues, testis and ovary, of the fish, *Pseudetroplus maculatus*.
2. Testicular oxidative stress could lead to impairment of sperm functional parameters and that could affect the reproductive potential of the fish.
3. Alteration in the antioxidant status in the ovary of the fish indicate the generation of reactive oxygen species, which could be the reason for the ovarian dysfunctions as alteration in ovarian steroidogenesis, vitellogenesis and atrophy of ovarian tissue.
4. Thus oxidative stress produced as a result of chlordecone exposure may lead to reproductive impairments in fish.
5. The destruction in normal reproductive capacity could result in serious consequences on the decline in fish population, which ultimately affect the biodiversity of aquatic ecosystem.

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CHAPTER 5

Effects of chlordecone on antioxidant status in non-reproductive tissues

Introduction

1.1 Generation of reactive metabolites in biological systems

The reactive metabolites are derived from oxygen or nitrogen and can be free oxygen radicals such as superoxide ($O_2^{\bullet-}$) or hydroxyl radical ($OH^{\bullet}$) or non-radicals like hydrogen peroxide (H_2O_2). Likewise, reactive metabolites are again classified as ions ($O_2^{\bullet-}$) or non-ions (H_2O_2). The reactive species derived from nitrogen are called reactive nitrogen species (RNS) and can be broadly classified as ions namely peroxynitrite ($ONOO^-$) or non-ions as nitric oxide ($NO^{\bullet}$) (Bolisetty and Jaimes, 2013). The reactive species either reactive oxygen species or reactive nitrogen species are thus formed at low levels during the implementation of physiological functions of the cell. In all aerobic organisms, general metabolism involves several oxidative processes especially, electron transport chain, which results in the generation of reactive oxygen species as an unavoidable byproduct that mediate biological functions such as apoptosis, necrosis and phagocytosis (Noori, 2012). Superoxide anions and hydrogen peroxides mediate the activity of several enzymes and are also used by phagocytic cells to kill bacteria (Pham-Huy *et al*., 2008). Generation of reactive oxygen species occurs continuously in all cells and it involves several mechanisms in different cell types that usually includes enzymatic and non-enzymatic reactions (Bedard and Krause, 2007; Orient *et al*., 2007). The reactive metabolites that are continuously generated in the cells are controlled by endogenous antioxidant enzymes, which include both non-enzymatic and enzymatic antioxidant systems which were discussed in detail in chapter 4.

1.2 Role of reactive metabolites in cells

There are several valuable functions performed by the reactive metabolites in the body and it was done mainly by maintaining the redox level in the cells. It play an important role as a mediator of detoxification processes, programmed cell death, phagocytosis of apoptotic cells, executioner of precancerous cells and infections. Apart from this, reactive metabolites are actively involved in signaling pathways to maintain cellular homeostasis in body. The production of highly reactive metabolites also regulates many metabolic and cellular processes such as differentiation,

proliferation, migration, immunity, wound healing and gene expression (Salganik, 2001).

1.3 Enzymatic sources of ROS

The enzymatic sources of ROS under subcellular levels include xanthine oxidoreductases, NADPH oxidases, cyclo-oxygenases (COX), lipoxygenases (LOX), nitric oxide synthases (NO synthases) and mitochondrial oxidases. The generation of reactive oxygen species occurs in mitochondria, cell membranes, peroxisomes, endoplasmic reticulum, lysosomes, and also during phagocytosis, apoptosis and auto-oxidation of small molecules (Han *et al.*, 2001; Lambeth, 2004).

1.3.1 Xanthine oxidoreductase (XOR)

The xanthine oxidoreductase generate reactive oxygen species by existing either as an oxidase that transfers reducing equivalents to oxygen, or as dehydrogenase that utilizes NAD or oxygen as the final electron acceptor (Krenitsky and Tuttle, 1978). Therefore, xanthine oxidoreductase exists as separate but in interconvertable forms such as xanthine dehydrogenase (XDH) and xanthine oxidase (XOD). Xanthine dehydrogenase is irreversibly transformed into xanthine oxidase by proteolysis and reversibly by oxidation of sulfhydryls along with the production of large amount of H_2O_2 and O_2. Generally, the enzyme catalyzes the oxidation of hypoxanthine to xanthine, and then into uric acid. XOR can also transform nitrates into nitrites and nitric oxide and then into highly reactive peroxynitrites (Cejkova *et al.*, 2002; Harrison, 2002). XDH and XOD are the rate-limiting enzymes in nucleic acid degradation and purine catabolism, which are also involved in the antimicrobial activity, iron metabolism, hepatic biotransformation etc. (Chung *et al.*, 1997). Furthermore, XDH and XOD are also responsible for certain pathological conditions such as xanthinuria, ethanol toxicity, influenza virus infection, atherosclerosis and gout (Kato *et al.*, 1990). XOR also act as a source of superoxide and hydrogen peroxide, which could exert protective or destructive effects that help to direct in generating nitric oxide and serve as a source of ROS intermediates (Harrison, 2002).

1.3.2 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

NADPH oxidase complex is a cluster of proteins where an electron from NADPH is transferred to molecular oxygen to produce superoxide thereby help to initiate the respiratory burst. It is the key step in immune defense against bacterial and fungal pathogens. Thus the enzyme is also otherwise known as respiratory burst oxidase. Generally the enzyme is present in the plasma membrane of phagocytic cells, especially in neutrophils that plays an important role in host defenses against invading microbes by generating superoxides (El-Benna *et al*., 2005). In the same manner, fibroblast, endothelial cells, mesangial cells, chondrocytes, osteoclast, and smooth muscles also produce superoxides with the help of NADPH-like oxidase activated by hormones and cytokines (Kerr *et al*., 1999). Apart from the host defense mechanism, NADPH oxidase also plays a vital role in cell signaling (Kerr *et al*., 1999).

1.3.3 Cytochrome P450 oxidase

Cytochrome P450 oxidase are members of hemoprotein found associated in the inner membrane of mitochondria or in the endoplasmic reticulum of cells where it plays an important role in the metabolism of several endogenous and exogenous compounds. Among all species of fish, about 137 genes that encode cytochrome P450 have been identified and are classified into 18 cytochrome P450 families (Uno *et al*., 2012). It is known to mediate the metabolism of cholesterol, steroids and other hormones, catabolism of bile acids, arachidonic acid and eicosanoids, hydroxylation of vitamin D3, retinoid acid by catalyzing intramolecular transfer of oxygen. In this process, one part of oxygen is reduced to superoxide anions (Keshari *et al*., 2015).

1.3.4 Monoamine oxidase

Monoamine oxidase is a key enzyme involved in the inactivation of neurotransmitters such as serotonin, dopamine and nor-adrenaline. It is the haem containing enzyme found within the outer membrane of mitochondria that catalyzes oxidative deamination of amines and produces hydrogen peroxide, which increases

the steady state concentrations of reactive oxygen species within the mitochondrial matrix and cytosol (Cadenas and Davies, 2000).

1.4 Production of ROS in cells

1.4.1 Mitochondrial generation of ROS

The mitochondrial electron transport chain (ETC) has been regarded as one of the major cellular producers of reactive oxygen species (Turrens, 2003; Andreyev *et al*., 2005). Generally, mitochondria consume over 90% of the cellular oxygen to enable oxidative phosphorylation and ATP synthesis (Brown, 1992; Han *et al*., 2001). The mitochondrial electron transport chain contains several complexes such as complex I, II, III and IV, containing multiple redox centers. The complexes are equally involved to facilitate the transfer of electrons from complex I (NADH oxidoreductase) to complex IV (cytochrome c oxidase), the ultimate electron acceptor, where the molecular oxygen is reduced by four electrons to water (Quinlan *et al*., 2013). During this process, some of the electrons passing through the mitochondrial electron transport chain, leak out to molecular oxygen or could result in the premature single electron reduction of molecular oxygen for the formation of superoxide. The superoxide thus formed is rapidly dismutated by the mitochondrial superoxide dismutase (Mn-SOD) to hydrogen peroxide (Boveris and Cadenas, 1975). *In vitro* mitochondrial electron transport chain converts around 2% of the oxygen consumed to univalently reduced superoxide anions, which is then transformed to hydrogen peroxide and hydroxyl ions (St-Pierre *et al.,* 2002).

Studies have shown that complex I and complex III of the electron-transport chain are the major sites for ROS production (Murphy, 2009; Brand, 2010). The complex I has two sites namely the flavin in the NADH-oxidizing site and the ubiquinone-reducing site that translocates four protons (H^+) across the membrane, thus producing a proton gradient (Treberg *et al*., 2011). Several sites between the flavin complex and the quinone site have been demonstrated to generate superoxide radicals within complex I (Herrero and Barja, 2000; Genova *et al*., 2003). Complex III ($CoQH_2$-cytochrome *c* reductase) has two quinol redox centers namely, the Qo center that are oriented towards the inter-membrane space and the Qi center, which

are located in the inner mitochondrial membrane facing the mitochondrial matrix (Chen *et al*., 2003). Superoxide radicals generated at the Qo center is released into the inter-membrane space whereas superoxide produced at the Qi center possibly enter into the matrix (St-Pierre *et al*., 2002). Earlier studies also have reported that complex III is the predominant site for the production of reactive oxygen species because the ROS products are directed away from the antioxidant defenses of the matrix in contrast to complex I, where the oxidants are directly released in the proximity of antioxidant enzyme system (Chen *et al*., 2003).

Generally, mitochondria contains 11 different sources of reactive oxygen species which are further divided into two different subgroups namely NADH/NAD isopotential group and QH2/Q isopotential group, based on redox potential at which they produce free radicals (Quinlan *et al*., 2013; 2014). The NADH/NAD isopotential group consists of four different enzymes namely complex I, oxoglutarate dehydrogenase (ODH), pyruvate dehydrogenase (PDH) and branched-chain alpha-keto acid dehydrogenase (BCKDH) that produce free radicals in response to variations in the concentrations of NADH. Increase in the level of NADH/NAD results in the sharp increase of free radical production by all enzymes in the isopotential group, especially complex I, ODH and PDH (Quinlan *et al*., 2014). All these enzymes possess flavin as prosthetic group, FMN in complex I or FAD in the other three enzymes, which are able to produce either superoxide or hydrogen peroxide or both (Goncalves *et al*., 2014). Complex I produce only superoxide free radicals whereas other enzymes generate both superoxide free radicals and hydrogen peroxide that make 75% of the total production (Mailloux, 2015).

The QH2/Q isopotential group consists of 7 enzymes namely complex III, succinate dehydrogenase (SDH), electron-transfer flavoprotein namely ubiquinone oxidoreductase (ETFQO), proline dehydrogenase, dihydroorotate dehydrogenase, succinate-coenzyme Q reductase (SQR) and sn-glycerol-3-phosphate dehydrogenase (sn-G3PDH). Quinol (Q) is a key electron transfer junction for aerobic respiration, accepting electrons from complex I and II following NADH and succinate oxidation, and all the enzymes of QH2/Q isopotential group require reduction of Q to QH2 for

maintaining the activity (Gnaiger *et al*., 2009). Thus the different site-specific topologies are important for redox signaling in the cells such as hypoxic signaling and ER-stress (Quinlan *et al*., 2013).

1.4.2 Role of cytochrome P450 (CYP) in ROS production

The cytochrome P450 is a family of heme-thiolate enzyme system involved in the oxidative metabolism of a variety of endogenous and exogenous lipophilic compounds (Sevanian *et al*., 1990). It is responsible for the basic metabolic reactions such as fatty acid oxidations and breakdown of a large number of toxic xenobiotics and carcinogens that enter the body (Rendic and Di Carlo, 1997). CYP-dependent hepatic microsomal electron transport system contributes one of the endogenous sources of reactive oxygen species. The hepatic microsomal cytochrome P450 dependant electron transport system has NADPH and oxygen-dependent functions, in which a significant portion of electrons is diverted to either the formation of hydrogen peroxide or the oxidative transformation of endogenous substrates (Hildebrandt *et al*., 1978). CYP enzymes catalyze the metabolism of xenobiotics that involves oxygenation and simultaneous reduction of molecular oxygen. If the transfer of oxygen to a substrate is not tightly controlled, uncoupling occurs that leads to the formation of reactive oxygen species (Hrycay and Bandiera, 2015). The utilization of molecular oxygen for the combined oxidation of NADPH and target compounds makes the enzyme systems a potentially significant source of ROS. Cytochrome P450 mediated generation of ROS is firmly controlled by the regulation of gene transcription and by modulation of communications between protein constituents of the monooxygenase that affects its activity, coupling and stability. Any failure in the mechanisms is known to result in a burst of ROS production, leading to lipid peroxidation and oxidative stress. Consecutively, the oxidative stress is also known to down-regulate P450 levels by a variety of feedback mechanisms (Zangar *et al*., 2004).

1.4.3 Other sources of ROS production

Moreover, certain compounds like catecholamines that are naturally present in organism are the important source for the production of ROS under specific physiological states, leading to diseases and aging (McAnulty *et al*., 2003). Reactive oxygen species are also produced in the cytosol and peroxisomes by different oxidases such as tryptophan dioxigenase (Li *et al*., 2007), xanthine oxidase (Shmarakov and Marchenko, 2008) and cytochrome P450 reductase (Clejan and Cederbaum, 1989). Moreover, most of the xenobiotics undergo autooxidation leading to the production of ROS in substantial amounts (Lushchak, 2011).

Minute fluctuations in the steady-state concentration of these oxidants are balanced by the antioxidant defense system with the aid of different antioxidant enzymes and non-enzymatic antioxidants (Rahal *et al*., 2014). Additional reactive metabolites are selectively neutralized by body's antioxidant defensive mechanism, thereby creating equilibrium between pro-oxidants and antioxidants in a cell (Betteridge, 2000). Whereas, uncontrolled increases in the steady-state concentrations of the oxidants or any imbalance in the equilibrium lead to a condition called oxidative stress that causes detrimental effects on the life of the cell. Hence oxidative stress is a situation of disturbed tissue oxidation, involving enhanced intracellular and extracellular ROS production that mediates peroxidation of lipids, proteins and DNA, and often result in a general disturbance of the cellular redox balance (Valko *et al*., 2007).

Several xenobiotics are known to induce oxidative stress either by generation of reactive oxygen species or by inhibiting the antioxidant system (Lackner, 1998). The severity depends upon the chemical properties of the contaminant, the extent to which the free radicals generated that prevail along with other factors related to extra and intracellular environment, such as ambient oxygen levels, the endogenous redox related activities, pH and other ionic balance and the co-presence of other bioactive agents (Pagano *et al.*, 2003). Thus the present study evaluates the effects of chlordecone, one of the estrogenic xenobiotics, at two sublethal concentrations on the antioxidant status of non-reproductive tissues as gill, liver and brain of *Pseudetroplus maculatus* for short-term durations.

Review of Literature

Pesticides are the group of chemicals that directly disturb oxidative balance and are responsible for the imbalance in homeostasis and directly linked to chronic diseases. According to the recent toxicological researches, one of the possible mechanisms of pesticide toxicity is the induction of oxidative stress. Exposure to pesticides results in the generation of reactive oxygen species that ultimately suppress the antioxidant levels leading to failure of defensive system against the oxidative damage. Mostly the stimulation of oxidative stress is targeted through the proteins, lipids and nucleic acids of the cells or tissues altering the cell signaling pathways. Pesticides are known to alter the activity of free radical scavenging antioxidant enzymes and other non-enzymatic antioxidants in different tissues, thereby induce lipid peroxidation leading to oxidative stress in animals (Banerjee *et al*., 2001). Moreover, most of the pesticides are lipid soluble so that these compounds are stored in fatty tissues of the exposed animal, and repeated small exposures may result in large accumulation which enhances the degree of toxicity leading to severe pathophysiological consequences (Abdollahi *et al*., 2004). Several long-term health effects such as tumour formation, cardiovascular defects, nerve disorders, respiratory and renal defects, endocrine disruption, and reproductive dysfunctions are associated with the pesticides induced oxidative stress.

Toxicant induced stress responses are classified based on its effects and resistance as intoxication and detoxication signals. Intoxication signals manifest devastating phenomena whereas the detoxication signals are adaptive in nature and offer protection to the biological systems against the exposure to toxicant (Bhattachary, 2001). Thus different manifestations of antioxidant capacity and oxidative stress can be noticed depending on the intensity and duration of toxicant exposure as well as resistance of the exposed organism. All cells in eukaryotic organisms are equipped with powerful antioxidant enzymes and non-enzymatic antioxidants which prevent the uncontrolled formation of reactive oxygen species that are formed either endogenously or exogenously, or inhibit the reactions with biological structures (Halliwell and Gutteridge, 2007). The antioxidant potential may vary from species to species and, within the same species differs among different cell types and subcellular fractions (Sies, 1997). When a xenobiotic

molecule enters the body of an animal, it undergoes redox cycling and generates reactive oxygen species. As an adaptive response, the body produces more antioxidant enzymes in order to eliminate the undesired reactive metabolites. Increased activities of superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase and glucose-6-phosphate dehydrogenase has been reported in grey mullet collected from the Huelva Estuary, south Atlantic Spain contaminated with higher concentrations of metals, such as iron and copper suggesting the adaptive response of the fish against the contaminants (Rodriguez-Ariza *et al.*, 1993). Similarly, long-term exposure to paper mill effluents caused induction of glutathione peroxidase, catalase and glutathione S-transferase and non-enzymatic antioxidants as glutathione and metallothionein in gill, liver and kidney of freshwater fish *Channa punctatus* (Ahmad *et al.*, 2000). Exposure to cadmium has been shown to increase the activities of superoxide dismutase and catalase in the erythrocytes of goldfish after 7 and 15 days of treatment (Zikic *et al.*, 2001). Activities of hepatic antioxidant enzymes increased in *Oreochromis niloticus* when exposed to higher concentrations of the crude oil after 15 and 30 days (Gad, 2011). Chlordecone, the pesticide used for the present study has been shown to cause expression of genes involved in the production of catalase and superoxide dismutase in prawns after 12 and 24 h of exposure (Gaume *et al*., 2015).

Xenobiotics exposure has been shown to induce reactive oxygen species generation that overwhelms the activities of antioxidant defense system leading to oxidative stress, which is characterized by continuous damages to biomolecules as lipids, proteins and DNA (Storey, 1996). One of the commonly used pesticide, chlorpyrifos has been found to inhibit the activities of superoxide dismutase, catalase and glutathione reductase along with induction of lipid peroxidation in the brain, liver and kidney of mosquito fish, *Gambusia affinis* (Kavitha and Rao, 2008). Rainbow trout, when exposed to propiconazole decreased activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and reduced glutathione content in the brain tissue (Li *et al.*, 2010). The glyphosate-based herbicide roundup transorb exposure caused reduction in superoxide dismutase, catalase and glutathione-S-transferase activities in the liver along with inhibition of

acetylcholinesterase in the brain and muscle of neotropical fish *Prochilodus lineatus* (Modesto and Martinez, 2010).

Fish gill is the first and foremost target of various pollutants in aquatic ecosystem, which is directly exposed to contaminated waters. The toxicants penetrate through the thin epithelial cells of gills where it either accumulate as such or enter into internal parts of the body and cause toxic effects (Roberts, 1978). Most of the aquatic pollutants are known to induce oxidative stress in gill by altering the activities of antioxidant enzymes and induction of lipid peroxidation. It has been reported that lipid peroxidation induced oxidative stress varies among the fish species and organs, which is usually indicated by the estimation of thiobarbituric acid-reactive substances (TBARS) (Oakes and Van der Kraak, 2003). Exposure to metal mixture of copper, cadmium, iron and nickel caused alterations in the activities of superoxide dismutase, catalase and glutathione S-transferase along with induction of lipid peroxidation and histological impairments in the gill tissue of *Channa punctatus* (Pandey *et al*., 2008). The commonly used pesticide, malathion at 0.05 mg/L has been shown to alter the activities of catalase, gluthatione peroxidase and glutathione reductase along with increase in the level of lipid peroxidation in gill, kidney and intestine of *Carassius auratus gibelio* within first day of exposure (Huculeci *et al*., 2009).

One of the pyrethroid insecticides, deltamethrin at 0.75 μg/L concentration have been shown to decrease the activity of catalase and increase the level of lipid peroxidation in gill, liver and kidney of cat fish after 48 h of exposure (Amin and Hashem, 2012). One of the most commonly used non-steroidal anti-inflammatory drugs diclofenac when exposed to common carp for short-term duration caused alterations in the activities of antioxidant enzymes and induced lipid peroxidation resulting in oxidative stress in the gill and liver tissues (Islas-Flores *et al*., 2013). *Labeo rohita*, when exposed to chromium has been shown to induce superoxide dismutase, catalase and glutathione reductase in gill, liver, brain and muscle tissues (Kumari *et al*., 2014). Dimethoate exposure has been shown to decrease the activities of SOD and GSH along with concomitant induction of lipid peroxidation in gill and liver tissues of zebrafish (Ansari and Ansari, 2014). Similar results were

observed when the catfish *Heteropneustes fossilis* exposed to two different sublethal concentrations of sodium fluoride in the gill, liver and kidney tissues (Singh and Tripathi, 2016). Sublethal exposure to chlorpyrifos has been shown to decrease the activities of SOD, CAT and GSH with concomitant induction of lipid peroxidation in the gill, liver and kidney of *Ctenopharyngodon idellus* after 15, 30 and 60 days of exposures (Kaur and Jindal, 2017).

The principal role of liver tissue in biochemical transformation of pollutants makes it more sensitive to environmental pollutant. Hepatic impairment is a common trend induced by various environmental contaminants and the variation is based on the intensity and duration of contaminant exposure. *Cyprinus carpio*, when exposed to sublethal concentrations of heavy metals such as cadmium, lead, nickel, and chromium has been shown to increase the activities of superoxide dismutase, catalase and glutathione peroxidase in the liver and kidney after 32 days (Vinodhini and Narayanan, 2009). Treatment of uranium at different concentrations has been shown to decrease the activities of superoxide dismutase and catalase in the liver of rainbow trout (Buet *et al*., 2005). Acute sublethal exposure to crude oil caused concentration and time-dependant induction of SOD, CAT and glutathione S-transferase enzymes in the liver of *Oreochromis niloticus* (Gad, 2011). Similarly, chronic sublethal exposure to bisphenol A has been shown to induce the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, and the content of reduced glutathione in the liver of medaka (Minghong *et al*., 2011).

In another study, when the freshwater fish, *Pangasius hypophthalmus* exposed to tannery effluent from tanning industry has been shown to cause inhibition in the activities of catalase, superoxide dismutase and reduced glutathione in the liver (Priya *et al*., 2012). The pesticide, beta-cypermethrin exposure caused increase in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase at 7 and 15 days but caused noticeable inhibition of the enzyme activities at 30 days in the liver of zebrafish (Mu *et al*., 2014). One of the pesticides, nitenpyram at different concentrations, 0.6, 1.2, 2.5, and 5.0 mg/ L has been shown to inhibit the activities of superoxide dismutase and catalase along with

increased malondialdehyde content in the liver of zebrafish after long-term treatment (Yan *et al*., 2015).

Usually, the brain tissue contains huge amounts of polyunsaturated fatty acids, which are predominantly susceptible to free radical attack that makes it more sensitive to contaminants including pesticides (Sahin and Gumuslu, 2004). Depletion in GSH content with apparent inhibition in SOD activity and induction of lipid peroxidation has been observed in the brain of Chinese rare minnow when exposed chronically at different concentrations of hexabromocyclododecane (Zhang *et al*., 2008). The acute exposure to propiconazole caused a significant decrease in the activity of SOD, CAT, GPx, and GR with concomitant induction of lipid peroxidation in the brain of rainbow trout (Li *et al*., 2011). The synthetic pyrethroid, cypermethrin treatment decreased the activity of catalase and increased the level of malondialdehyde, however, the effects has been found reversed by the addition of a phenolic compound, propolis in the brain of rainbow trout (Kakoolaki *et al*., 2013). Chronic exposure to atrazine has been shown to increase the activities of catalase and superoxide dismutase along with the decrease in the activity of acetylcholinesterase enzyme in the brain of zebrafish (Al-Sawafi and Yan, 2013). Exposure to cadmium in zebrafish increased the activities of catalase, superoxide dismutase whereas inhibited the activity of acetylcholinesterase in brain and muscle (Al-Sawafi and Yan, 2013). Endosulfan exposure has been shown to cause the induction of superoxide dismutase, catalase, glutathione reductase, reduced glutathione and glutathione peroxidase together with increased level of malondialdehyde content in the brain, gill, liver and muscle of *Labeo rohita* (Ullah *et al*., 2016).

The level of antioxidant enzymes have been extensively used as an early warning indicator of lake pollution (Lin *et al*., 2001). One of the comparative studies reported that *Mugil cephalus* showed increased activities of superoxide dismutase and catalase together with concomitant increased in the level of lipid peroxidation than the fish, *Platichthys flesus* when collected from Douro Estuary, Portugal, contaminated with pesticides (Ferreira *et al*., 2005). Grey mullets sampled from highly polluted Ennore estuary, Chennai showed decreased activities of hepatic

superoxide dismutase and catalase along with increased rate of lipid peroxidation (Padmini *et al*., 2008). Nile tilapia collected from different polluted sites of the Lake Mariut and Lake Edku in Egypt showed decreased activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase along with induction of lipid peroxidation in the gill tissue (Abdel-Moneim *et al*., 2012). The activities of superoxide dismutase, catalase, glutathione S-transferase, glutathione reductase and glucose 6-phosphate dehydrogenase increased along with the induction of lipid peroxidation in the liver of *Barbus bocagei* collected from polluted regions of the river Vizela, Portugal (Peixoto *et al*., 2013).

The activities of superoxide dismutase, catalase and reduced glutathione content were altered in liver, kidney and brain tissues of *Channa striatus* and *Heteropneustes fossilis* inhabiting Kali River of northern India, contaminated with heavy metals chromium, nickel, lead and cadmium (Fatima *et al*., 2015). Cypermethrin have been shown to induce cell death of macrophages through cell cycle arrest and oxidative stress mediated apoptosis (Huang *et al*., 2016). In another study, *Siganus canaliculatus* caught from Jeddah and Yanbu coast contaminated with oil hydrocarbons showed increased expressions of superoxide dismutase, catalase, gutathine reductase and glutathione peroxidase genes in gill, liver, brain and skin (Afifi *et al*., 2017). Phorate induced oxidative stress that led to DNA damage and enhanced expression of apoptotic genes-tumor suppressor (p53), apoptotic peptidase activating factor-1 (apaf-1) in *Channa punctatus* (Ratn *et al*., 2017). Studies have shown that oxidative stress in different tissues induce long-term health effects in fish such as carcinogenesis, neuro-degeneration, respiratory, cardiovascular, renal, endocrine and reproductive problems (DSouza, 2017). An overview of the literatures reviewed ensured that the imbalance between the free radicals generated and scavenging effects of antioxidants could result in the induction of oxidative stress. The consequence of chlordecone on the antioxidant status of non-reproductive tissues was evaluated in the present study by exposing the fish at different sublethal concentration in different durations, which to some extent, may provide basic knowledge on assessing the risk of chlordecone in the fish, *Pseudetroplus maculatus*.

Materials and Methods

3.1 Grouping of animal and treatment

After acclimatization of fish in the laboratory condition, they were grouped into various groups in different tanks according to the selected test concentrations and test durations by maintaining ten animals per group along with the control groups. The median lethal concentration of chlordecone for 96h (LC_{50}–96 h) in *Pseudetroplus maculatus* was determined as 35 µg/ L (Asifa and Chitra, 2015) by probit analysis. In the experiment, two sublethal concentrations such as 3.5µg/ L and 7µg/ L ($1/10^{th}$ and $1/5^{th}$ of LC_{50}) of chlordecone, dissolved in 1% DMSO (Dimethyl sulfoxide) was selected based on the LC_{50}–96 h value and exposed for three durations such as 24, 72 and 96h.

3.2 Collection of tissues

At the end of every treatment period, each fish was captured gently using a small dip net without stress to the animal. Fish was then sacrificed by decapitation followed by the dissection of gill, liver and brain tissues, cleared from mucous, debris and blood cells, weighed and used for further analysis.

3.3 Preparation of tissue homogenates for biochemical analysis

Gill, liver and brain tissues collected from each control and treatment groups were stored at 4ºC until further processing. Tissue homogenates (1% w/ v) were prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 800*g* for 15 min at 4ºC to obtain the supernatant, which was then used for the biochemical analyses. In supernatant of crude tissue homogenates, protein was determined by the method of Lowry *et al* (1951) with BSA as the standard. Activities of superoxide dismutase (Marklund and Marklund, 1974), catalase (Claiborne, 1985), glutathione reductase (Carlberg and Mannervik, 1985), levels of hydrogen peroxide generation (Pick and Keisari, 1981), lipid peroxidation (Ohkawa *et al*., 1979), activities of alkaline phosphatase (Bessey *et al*., 1946) and acetylcholinesterase (Ellman *et al*., 1961) were analysed.

3.4 Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 19.0) was used to analyse the significance of the results. One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test was performed in the experiment. Differences were considered to be significant at $p<0.05$ against the control groups and are denoted as asterisk (*) symbol in the Figures and the data are presented as mean±SD for ten animals per group. All biochemical estimations were carried out in duplicate to avoid statistical errors.

Results

4.1 Effects of chlordecone on the tissue weights of the fish, *Pseudetroplus maculatus*

Chlordecone exposure at 7µg/ L concentration after 96 h showed significant ($P<0.05$) reduction in the hepatosomatic index as well as the weights of gill and brain when compared to the control groups (Figures 1-3). Fish when exposed to 1% DMSO (vehicle control) showed no significant changes in the weights of any tissues and was found similar to that of the negative control group (Figures 1-3).

4.2 Effects of chlordecone on the antioxidant status in the gill of the fish

In gill tissue, chlordecone exposure at both sublethal concentrations showed significant ($P<0.05$) increase in the activities of superoxide dismutase, catalase and glutathione reductase in concentration-dependant manner when compared to the corresponding control groups (Figures 4-6). The levels of hydrogen peroxide generation and lipid peroxidation significantly ($P<0.05$) increased in the gill tissue at both concentrations than the control groups (Figures 7 and 8).

4.3 Effects of chlordecone on the antioxidant status in the liver of the fish

In the liver tissue, activity of superoxide dismutase was increased whereas the activities of catalase and glutathione reductase decreased with a concomitant increase in hydrogen peroxide generation and lipid peroxidation (Figures 9-13).

4.4 Effects of chlordecone on the antioxidant status in the brain of the fish

In brain tissue, a significant decrease in the activities of all antioxidant enzymes along with significant ($P<0.05$) increase in the levels of hydrogen peroxide and lipid peroxidation were observed at 7µg/L (Figures 14-18).

4.5 Effects of chlordecone on the tissue marker enzymes in the fish

The activity of alkaline phosphatase in gill and liver tissues and the activity of acetylcholinesterase enzyme in brain tissue showed concentration- and time-dependant significant ($P<0.05$) decrease when compared to the control groups (Figures 19-21).

Figure 1: **Effect of chlordecone on the weight of gill in the fish, *Pseudetroplus maculatus***

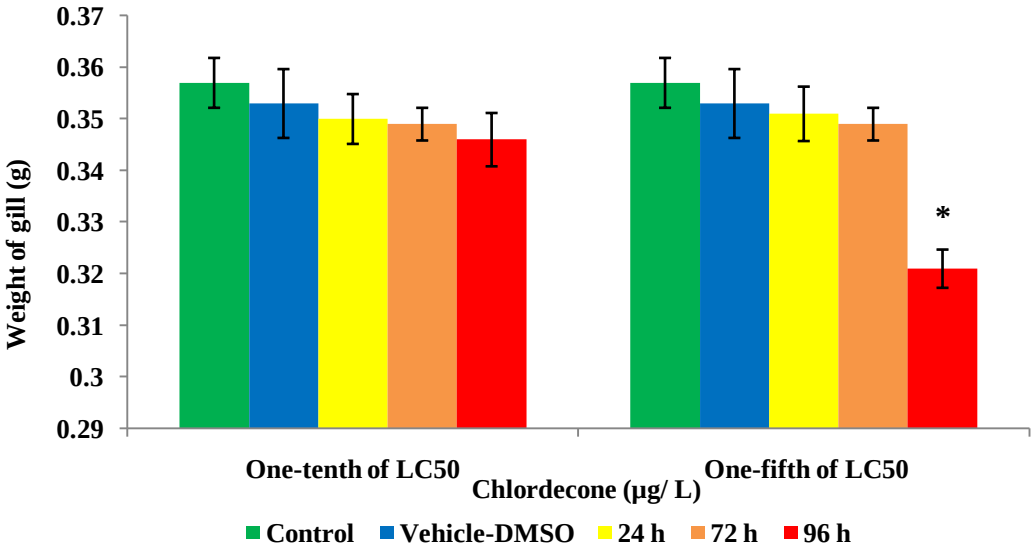


Figure 2: **Effect of chlordecone on the hepatosomatic index of the fish, *Pseudetroplus maculatus***

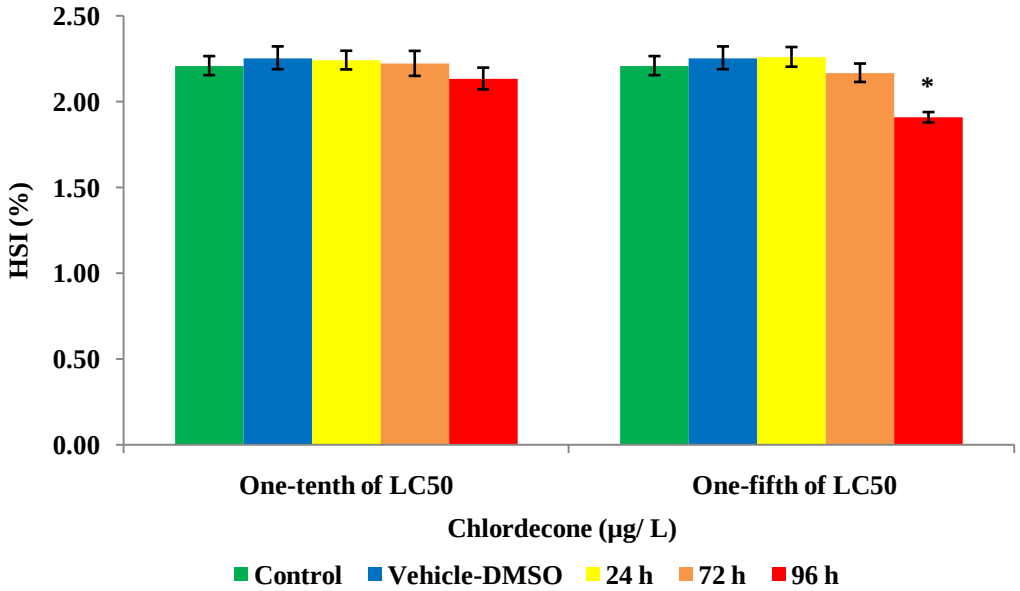


Figure 3: **Effect of chlordecone on the weight of brain of the fish, *Pseudetroplus maculatus***

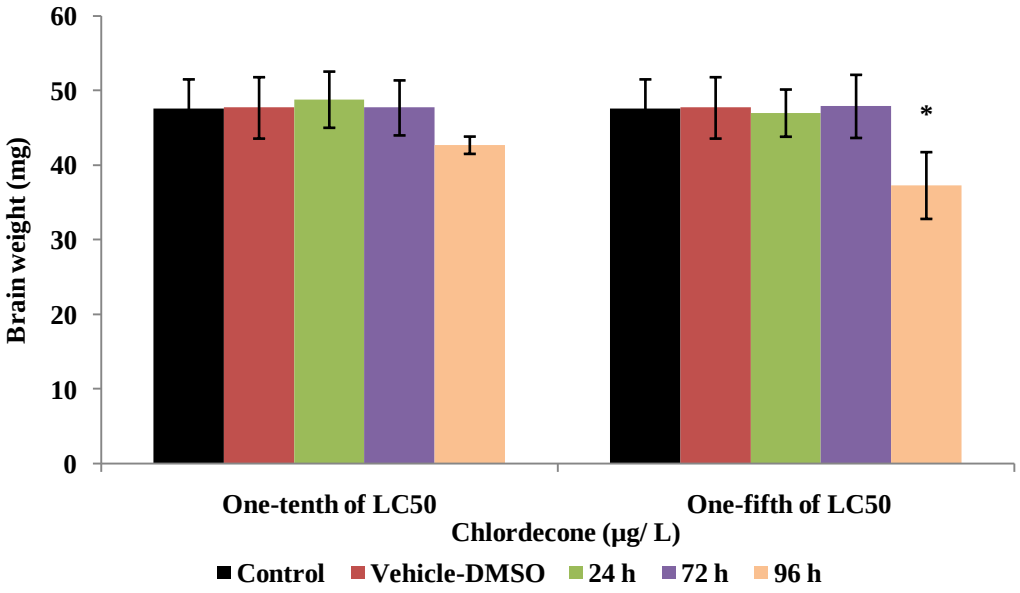


Figure 4: **Effect of chlordecone on the activity of superoxide dismutase in gill of the fish, *Pseudetroplus maculatus***

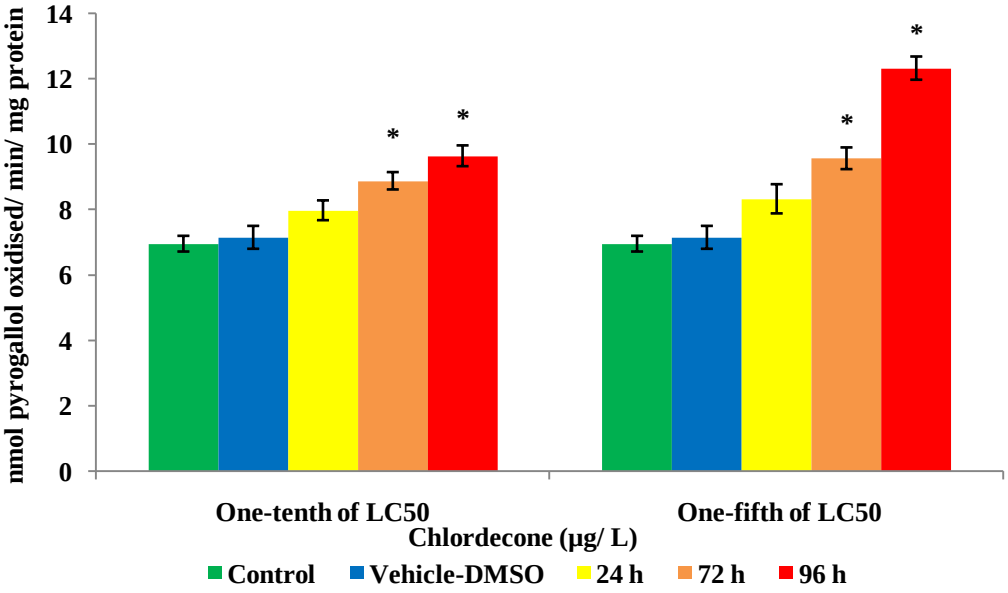


Figure 5: **Effect of chlordecone on the activity of catalase in gill of the fish, *Pseudetroplus maculatus***

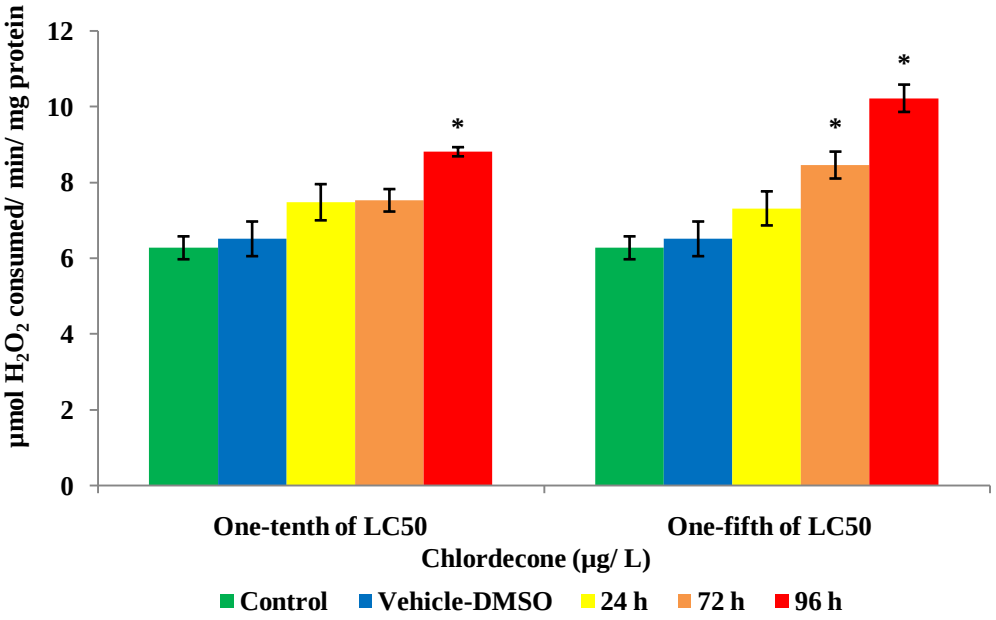


Figure 6: **Effect of chlordecone on the activity of glutathione reductase in gill of the fish, *Pseudetroplus maculatus***

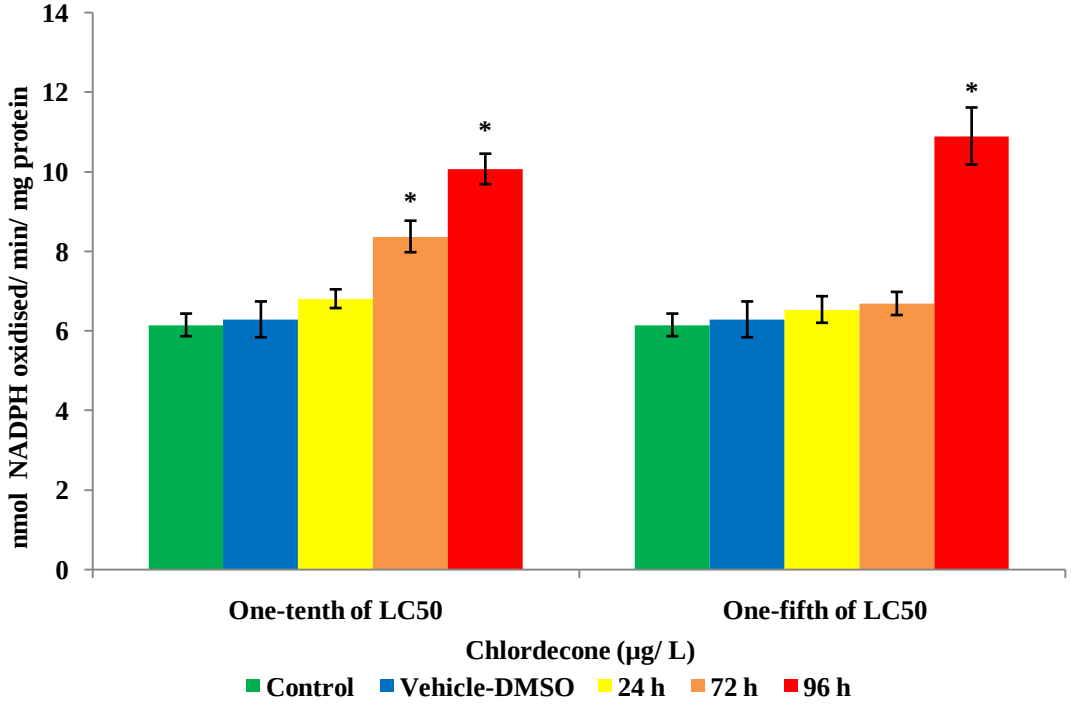


Figure 7: **Effect of chlordecone on the level of hydrogen peroxide generation in gill of the fish, *Pseudetroplus maculatus***

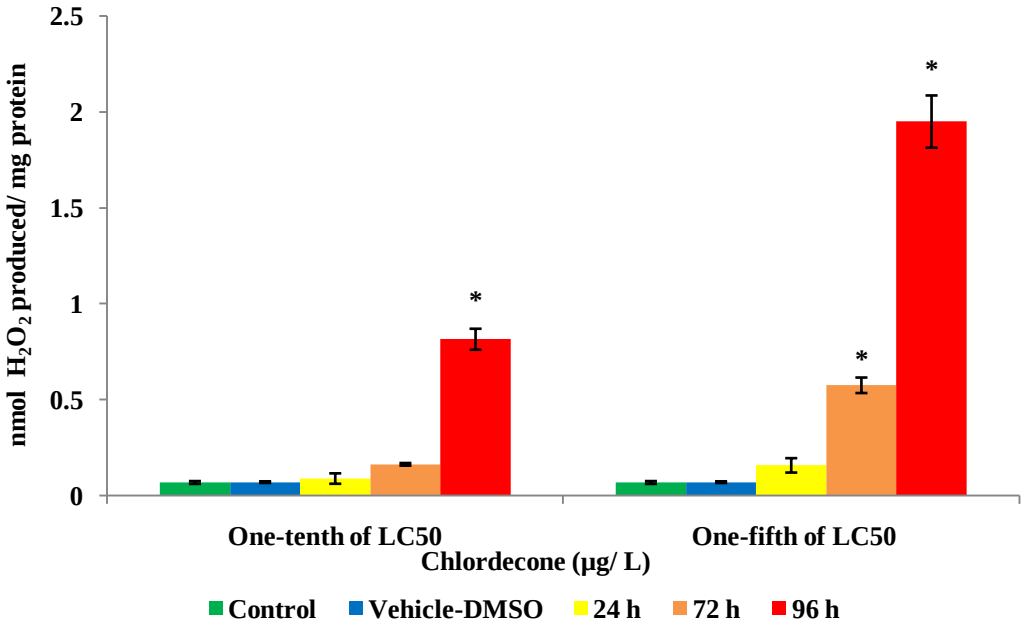


Figure 8: **Effect of chlordecone on the level of lipid peroxidation in gill of the fish, *Pseudetroplus maculatus***

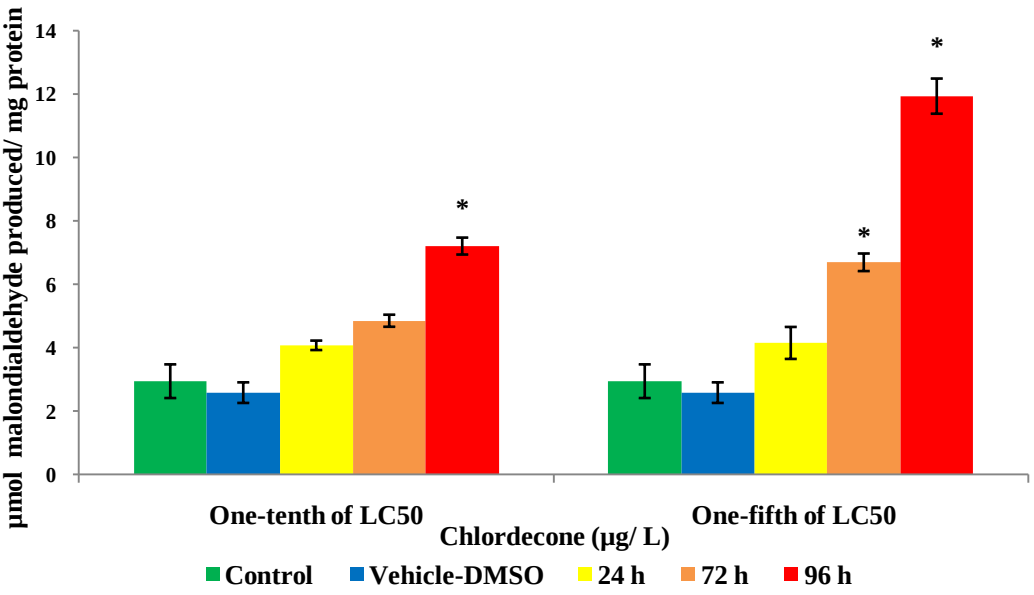


Figure 9: **Effect of chlordecone on the activity of superoxide dismutase in liver of the fish, *Pseudetroplus maculatus***

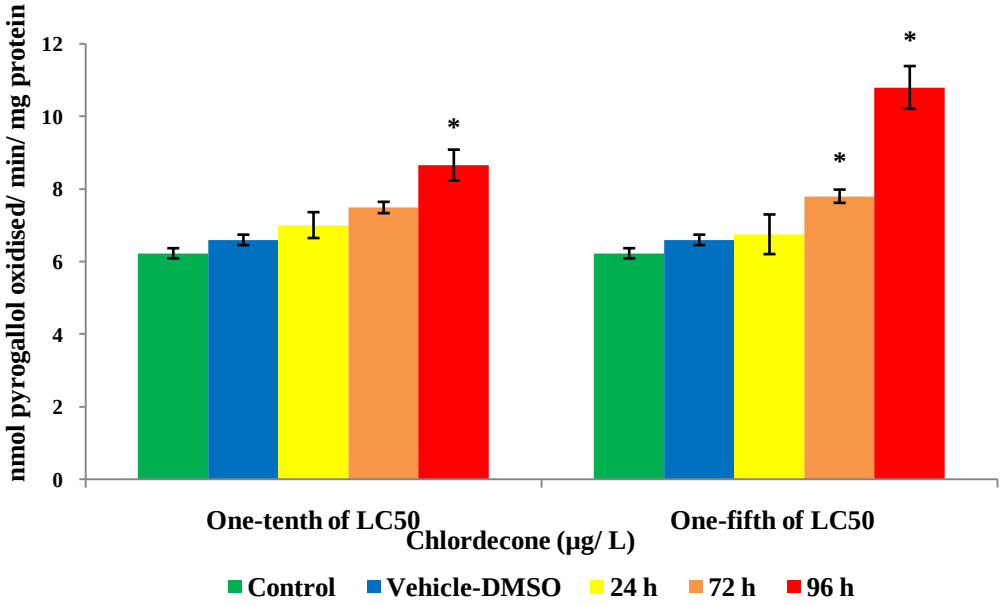


Figure 10: **Effect of chlordecone on the activity of catalase in liver of the fish, *Pseudetroplus maculatus***

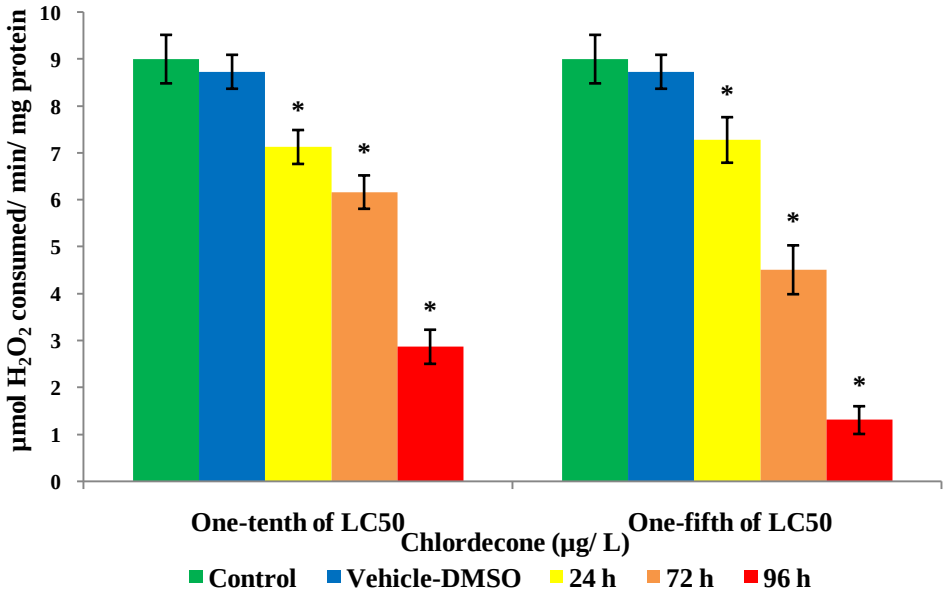


Figure 11: **Effect of chlordecone on the activity of glutathione reductase in liver of the fish, *Pseudetroplus maculatus***

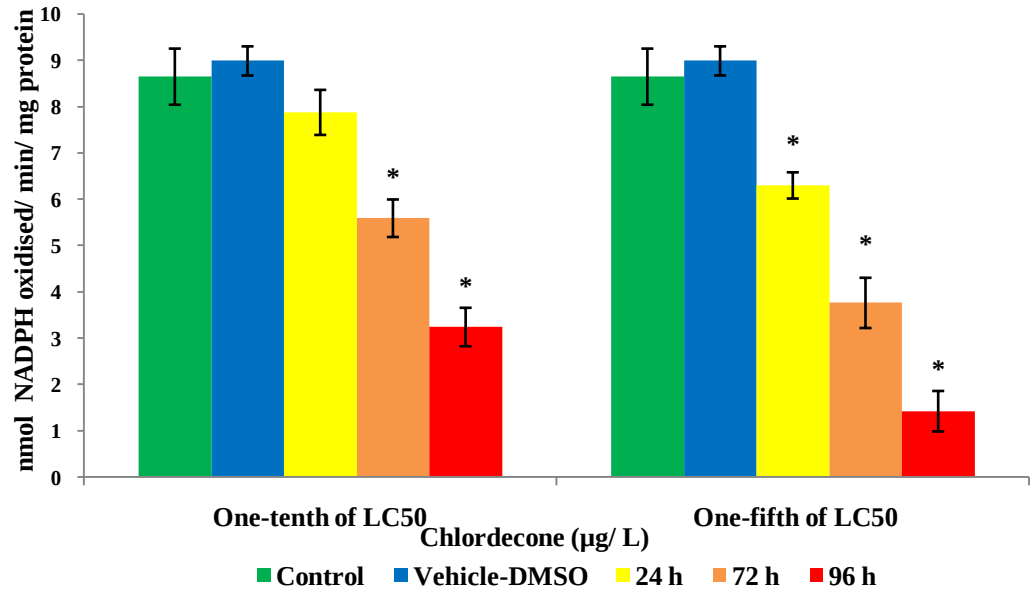


Figure 12: **Effect of chlordecone on the level of hydrogen peroxide generation in liver of the fish, *Pseudetroplus maculatus***

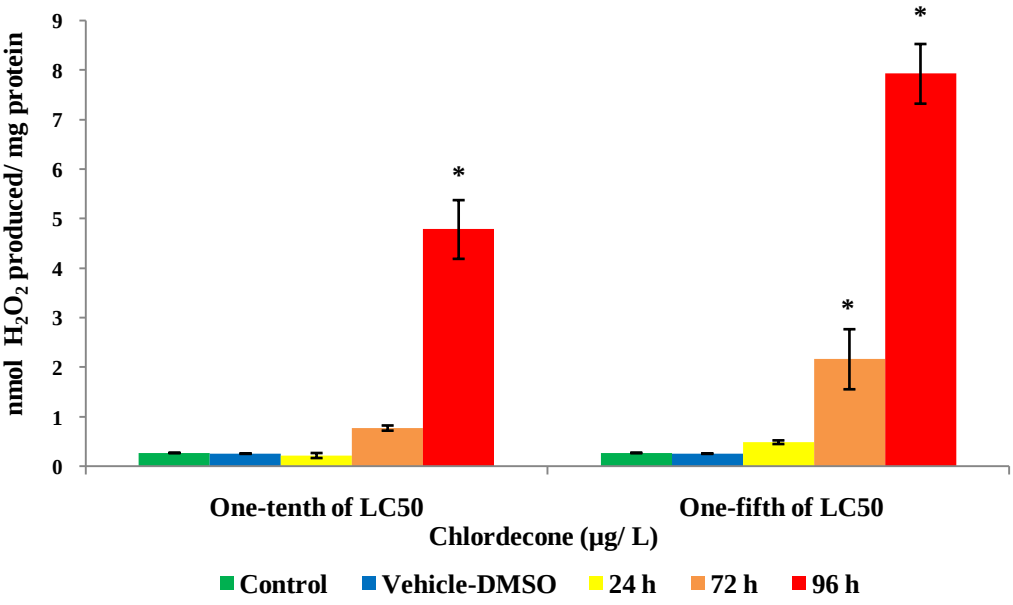


Figure 13: **Effect of chlordecone on the level of lipid peroxidation in liver of the fish, *Pseudetroplus maculatus***

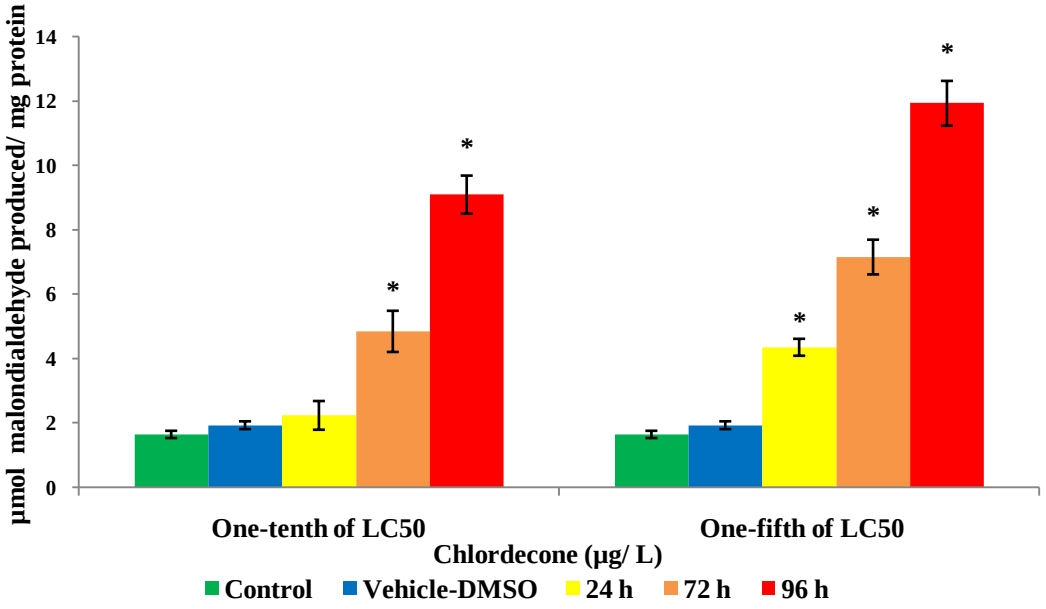


Figure 14: **Effect of chlordecone on the activity of superoxide dismutase in brain of the fish, *Pseudetroplus maculatus***

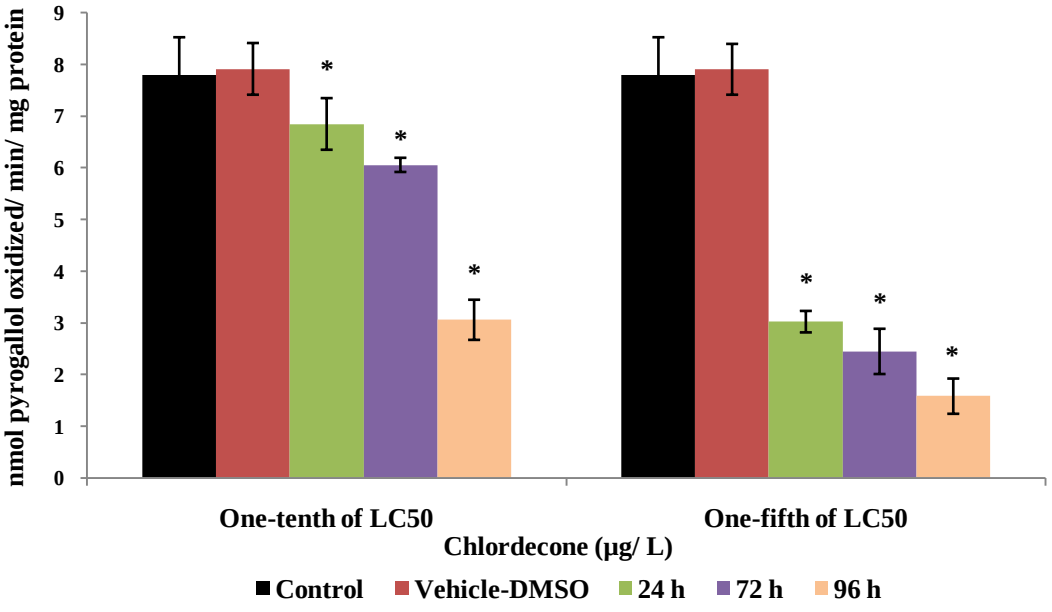


Figure 15: **Effect of chlordecone on the activity of catalase in brain of the fish, *Pseudetroplus maculatus***

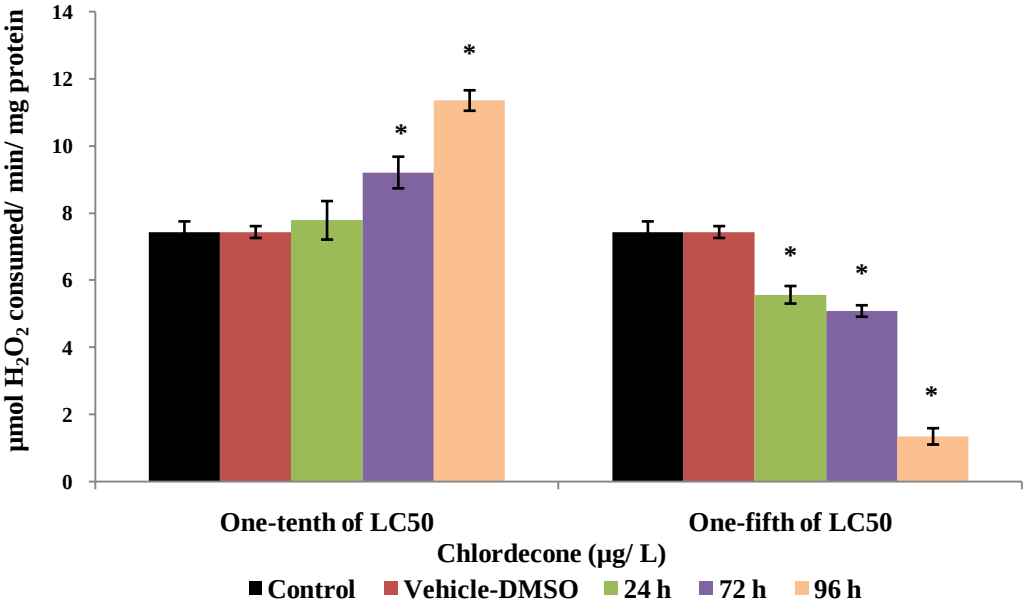


Figure 16: **Effect of chlordecone on the activity of glutathione reductase in brain of the fish, *Pseudetroplus maculatus***

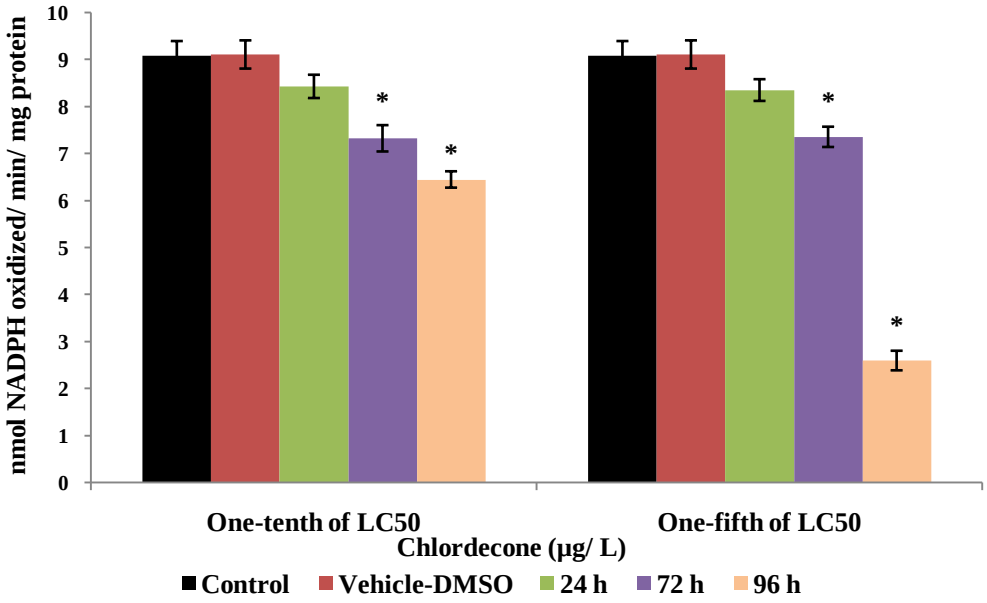


Figure 17: **Effect of chlordecone on the level of hydrogen peroxide generation in brain of the fish, *Pseudetroplus maculatus***

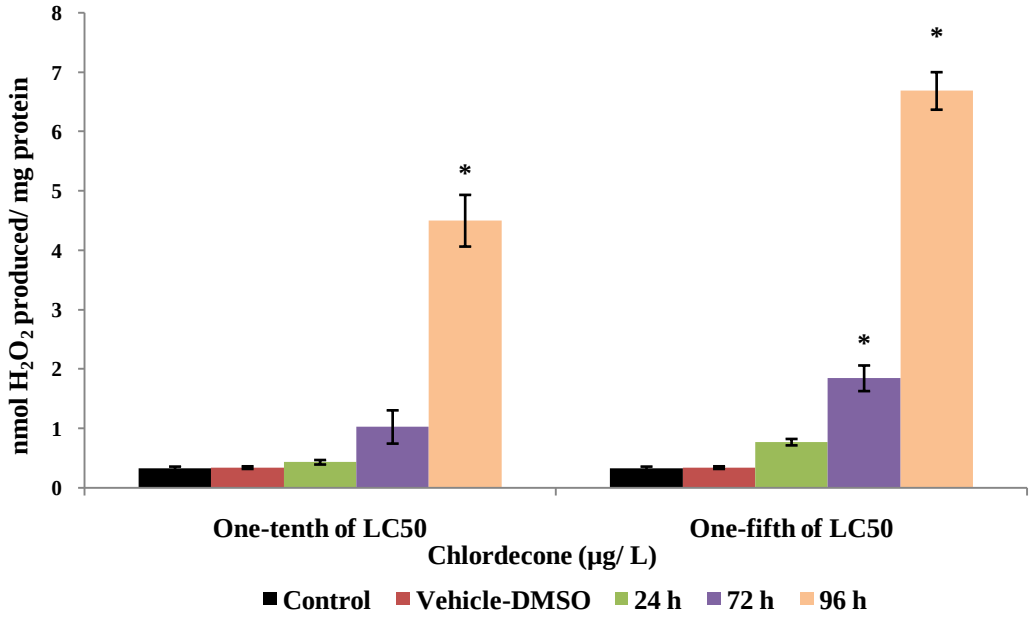


Figure 18: **Effect of chlordecone on the level of lipid peroxidation in brain of the fish, *Pseudetroplus maculatus***

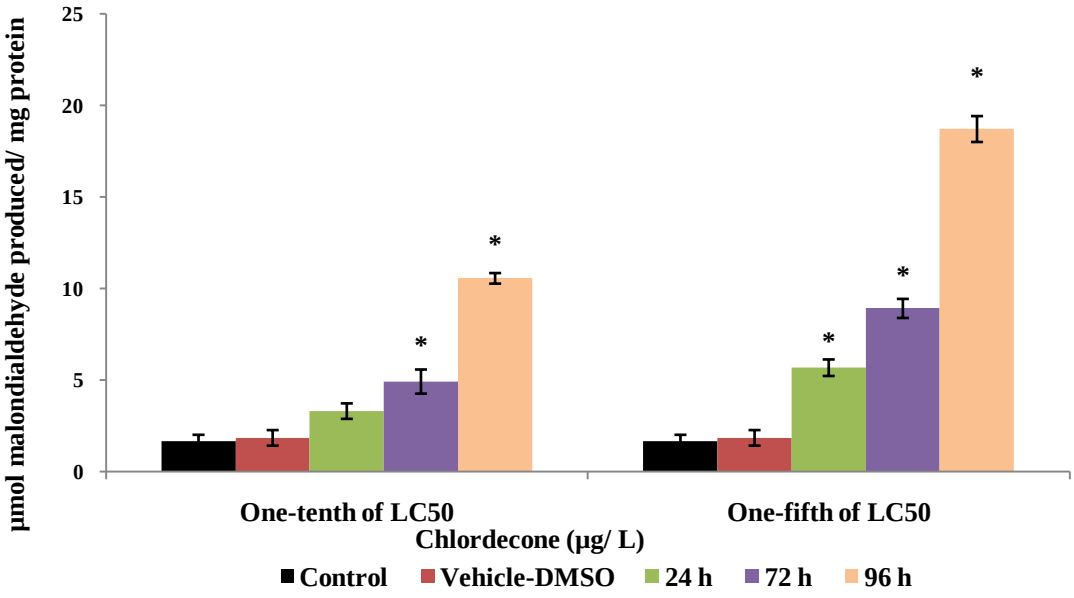


Figure 19: **Effect of chlordecone on the activity of alkaline phosphatase in gill of the fish, *Pseudetroplus maculatus***

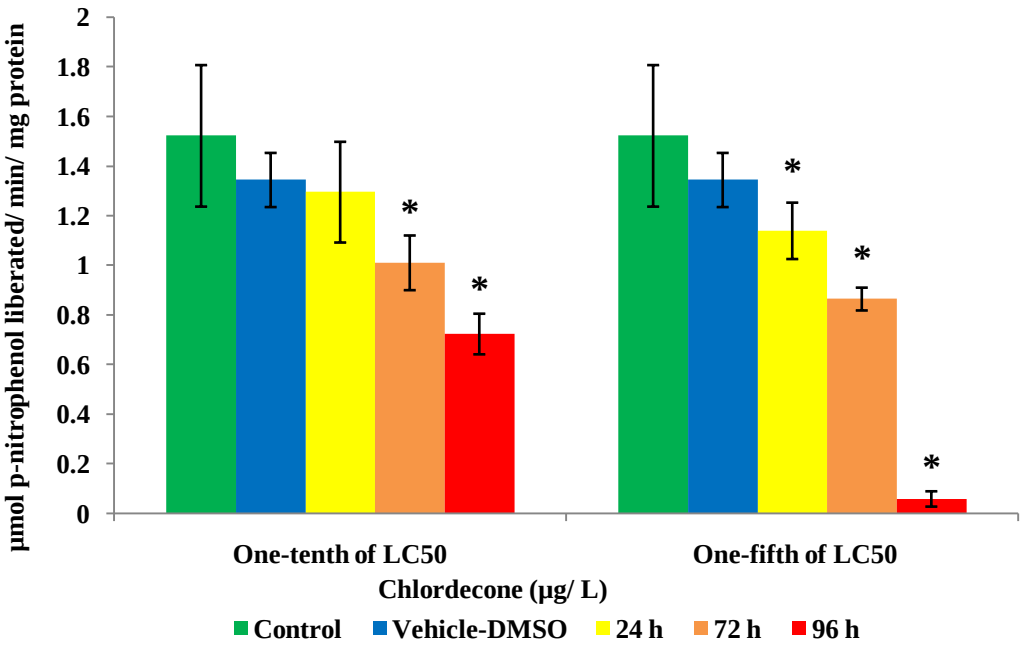


Figure 20: **Effect of chlordecone on the activity of alkaline phosphatase in liver of the fish, *Pseudetroplus maculatus***

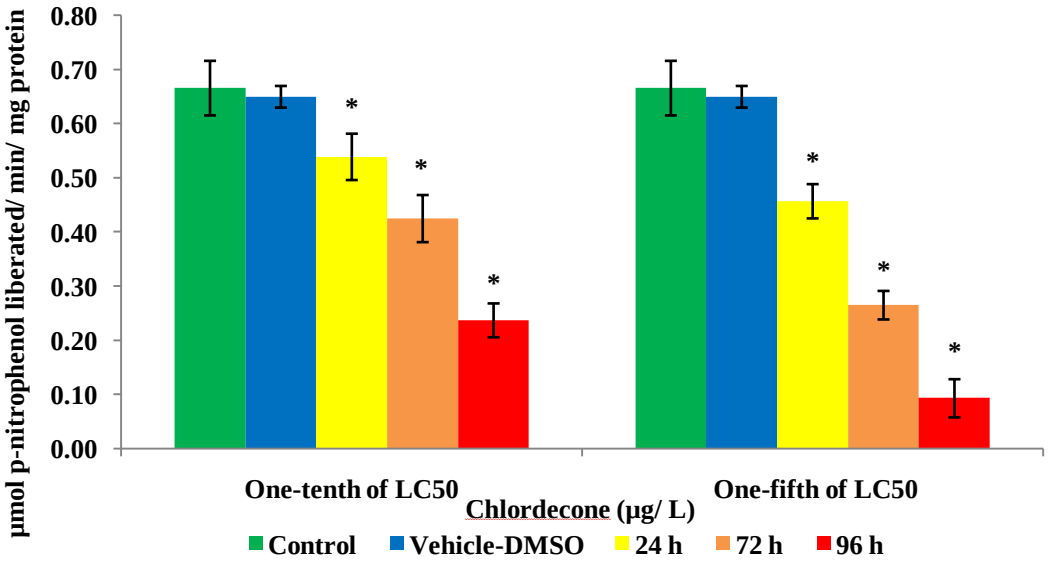
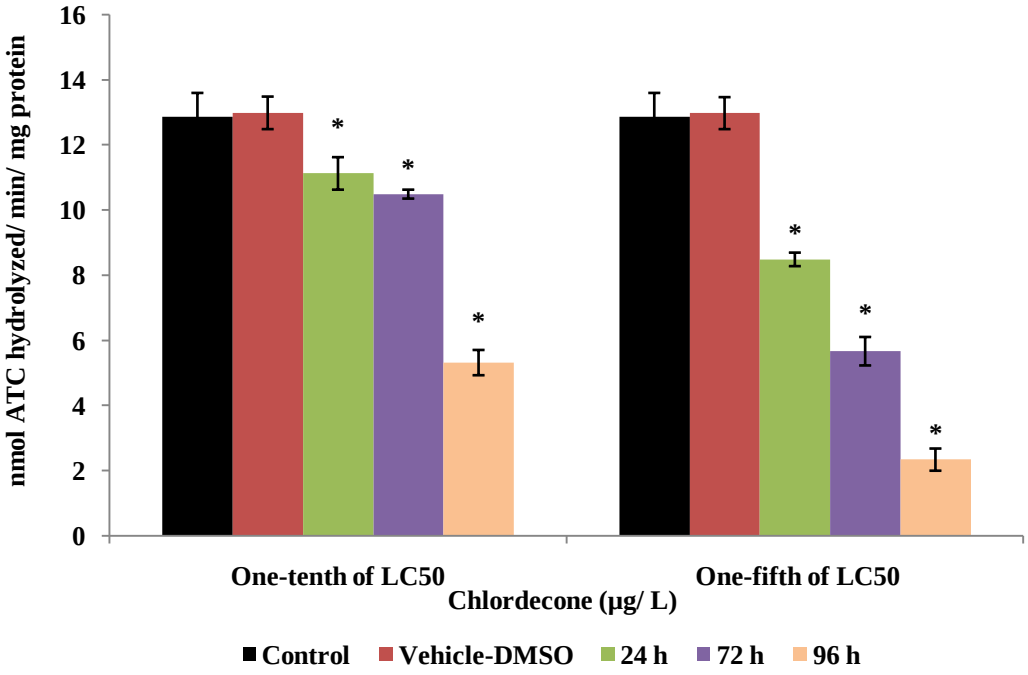


Figure 21: Effect of chlordecone on the activity of acetylcholinesterase in brain of the fish, *Pseudetroplus maculatus*



Discussion

5.1 Effects of chlordecone on tissue weights

Change in tissue weight is a rough but good and sensitive indicator to detect general toxicity in animals. In most of the toxicity studies, comparison of tissue weights of treated and untreated groups provide a useful tool to detect the toxic effect of the exposed chemical. Chlordecone exposure decreased the weights of gill, brain and the hepatosomatic index of *Pseudetroplus maculatus* at the end of 96 h only at one-fifth concentration of LC_{50} when compared with the control groups.

Gill is the primary organ exposed directly to the toxicant as it is in direct contact to the toxic environment. Gill tissue is most susceptible for the exposed contaminant and the present study showed decrease in the weight of gill which may be due to the direct effect of chlordecone. Brain weight is considered as least affected to the toxicity of contaminants. But the present result showed significant decrease in the brain weight after 96 h which reflect that the toxicant, chlordecone possess the ability to pass through blood-brain barrier. Liver weight was considered as sensitive parameter to predict general toxicity of the contaminant and it is a frequent target in toxicity studies. Organ-to-body weight ratio helps to stabilize the variability due to the nutritional status. Hence the decrease in the hepatosomatic index, the relative liver weight to the animal weight, signifies the toxicity of chlordecone. Thus the evaluation of tissue weight is widely used to diagnose the normal metabolic functions, physiological and histopathological changes that are likely to vary among the same or different species. The decrease in tissue weights could be due to atrophy or necrosis of tissues as a result of chlordecone intoxication which is discussed in chapter 7. The result was found in accordance to another study conducted in the same fish when exposed to chlorpyrifos at sublethal concentration for 96 h (Raibeemol and Chitra, 2017a, b).

5.2 Effects of chlordecone on gill antioxidant system

In fish, biochemical analyses are the sensitive parameter to detect potential adverse effects of environmental contaminants. The activities of various enzymes are considered to be sensitive biochemical indicators and are important parameters for testing water for the presence of toxicants (Gul *et al*., 2004). Gills are the

principal target tissue to aquatic pollutants as it is in continuous and constant contact with environment containing pollutants (Perry and Laurent, 1993). Gill performs multiple vital functions such as respiration, osmoregulation and excretion in fishes. Gill is particularly sensitive to various physical and chemical changes in the aquatic environment since the large surface area is exposed to the external environment thereby being the target for the contaminants in water (Mallatt, 1985). Thus when the aquatic environment is contaminated, gill tissue responds in advance than the kidney and liver tissues (Ahmad *et al.,* 2004; Santos *et al.,* 2004). Moreover, gills of fish are capable to accumulate chemicals that enter the body through other exposure routes as it occupies in between the venous and arterial circulation thus receiving nearly all of the cardiac output (Levine and Oris, 1999). The present study focused on the gill antioxidant system because the possible role of free radicals in generating toxicity to animals has received much attention in recent years.

Chlordecone exposure showed a significant increase in the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase at both concentrations after 72 and 96 h of exposure in the gill tissue of *P. maculatus*. The SOD-CAT system is the first line of defense for the removal of free radicals where it functions together and occurs in tandem (Pandey *et al.,* 2003). SOD catalyzes the biochemical reaction for the conversion of superoxide radicals to hydrogen peroxide (H_2O_2) whereas catalase removes the generated hydrogen peroxide concomitantly (Halliwell, 1994). The increase of SOD and CAT activities probably could be in response to the increased superoxide anions and H_2O_2 levels (John *et al.,* 2001). In particular, the increase of CAT activity may be due to the SOD-stimulated H_2O_2 production since CAT is responsible for the detoxification of hydrogen peroxide into water (Farombi *et al*., 2008). However, the gill antioxidant system of *P. maculatus* failed to eliminate the free radicals completely, which is revealed by the significant increase in hydrogen peroxide generation and concomitant increase in the level of lipid peroxidation. The present study employed thiobarbituric acid method for measuring the production of malondialdehyde in tissues, which is the end-product of lipid peroxidation. As the quantitative indicator of lipid peroxidation, this method is the most reliable indicator as it detects all existing malondialdehyde along with other

thiobarbituric end products (Baege and Aust, 1978). Therefore, the present results clearly indicate that chlordecone treatment stimulated reactive oxygen species production in gill though it attempted an adaptive response to get rid of free radicals by increasing the activities of antioxidant enzymes. Similar results have been observed when gold fish exposed to diesel oil for 40 days (Zhang *et al.,* 2004), Atlantic cod (*Codus morhua*) exposed to sea oil and alkylphenol for 15 days (Sturve *et al.,* 2006), *Oreochromis mossambicus* exposed to bisphenol A (Chitra and Sajitha, 2014) and *Oreochromis niloticus* exposed to phenanthrene for 14 days (Wenju *et al.,* 2009).

5.3 Effects of chlordecone on hepatic antioxidant system

Liver is often associated with metabolism, detoxification and biotransformation of xenobiotics and is a principal organ attacked by the effects of reactive oxygen species. Hepatotoxicity is therefore an important endpoint for assessing the toxic effects of pollutants in fish, especially targeted on pesticides exposure (Patil and David, 2009). The hepatic parenchymal cells are the primary cells subjected to oxidative stress induced injury in the liver. Within the parenchymal cells, reactive free radicals are mainly produced by mitochondria and peroxisomes. In addition, Kupffer cells, hepatic stellate cells and endothelial cells are more sensitive to oxidative stress-related molecules (Sanchez-Valle *et al.,* 2012). Moreover, hepatic biotransformation and electron transport chain contribute to the generation of reactive oxygen species in the liver tissue (Taylor *et al*., 1995). The present observation showed a significant increase in the activity of superoxide dismutase after chlordecone exposure in the liver of *P. maculatus*. The present result is in good accordance with the exposure to atrazine for 14 days in zebrafish that showed an increase in the activity of SOD in liver tissues (Jin *et al.,* 2010). However, chlordecone exposure decreased the activities of hepatic catalase and glutathione reductase when compared with the control groups. One of our previous studies reported that acute sublethal concentrations of nonylphenol inhibited the activities of SOD, CAT and GR in the liver of *Etroplus maculatus* (Asifa and Chitra, 2016a). The induction of superoxide dismutase could be a defensive mechanism of

hepatocytes to convert superoxide radicals to hydrogen peroxide (Alves *et al.,* 2002), but catalase was unable to remove the hydrogen peroxide generated. The justification of the decrease in catalase activity could be the failure of enzyme to cope up with the sudden flux of superoxide radicals induced by the pollutant (Ahmad *et al.,* 2000). The inhibition of catalase enzyme lead to failure in scavenging hydrogen peroxide from the hepatocytes, which is evidenced by 25-40 fold increase in the generation of hydrogen peroxide after 96 h of chlordecone exposure. Hydrogen peroxide generation ultimately leads to the induction of lipid peroxidation, which is the oxidative degradation of lipids resulting in cell damage. In the present study, the activity of glutathione reductase decreased in time and concentration-dependent manner which indicate the failure of the enzyme to convert oxidized glutathione to reduced glutathione thereby the resistance to oxidative stress and maintenance of reducing environment of the cell is prevented. The present result coincides with one of the previous studies in our laboratory in which fullerene exposed for short-term durations increased the activity of SOD and decreased the activities of catalase and glutathione reductase along with induction of hydrogen peroxide and lipid peroxidation in the liver of *P. maculatus* (Sumi and Chitra, 2017a). Similarly, exposure to silicon dioxide nanoparticles showed alteration in the activities of antioxidant enzymes in liver tissues of *O. mossambicus* when exposed for 96 h (Vidya and Chitra, 2015). Thus in the present study a state of oxidative stress is formed in the hepatocytes of fish when the generation of reactive oxygen species is relatively higher than the rate of elimination as a result of chlordecone exposure.

5.4 Effects of chlordecone on brain antioxidant system

Brain is the principal organ which is highly rich in easily oxidized polyunsaturated fatty acids, also an excellent consumer of high levels of energy and physiological oxygen but with poor antioxidant defense mechanisms, which makes it predominantly susceptible to oxidative stress (Migliore and Coppede, 2009). Brain tissue needs more energy for various physiological functions and to maintain mitochondrial oxidative metabolism, thus it is more prone to reactive oxygen species

(Mates, 2000; Soengas and Aldegunde, 2002). Chlordecone exposure caused a significant decrease in the activity of SOD and GR at both sublethal concentrations. But a significant induction of CAT activity was observed at lower concentrations of chlordecone after 96 h of treatment, and when the concentration increases, its activity declined in a time-dependent manner. Chlordecone exposure increased the levels of hydrogen peroxide and lipid peroxidation in brain tissue. Similar results were reported when the fish was exposed to one of the nanoparticles, fullerene for 96 h (Sumi and Chitra, 2017b). Similarly, chronic carbamazepine treatment has been reported to cause decrease in the activities of superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and level of GSH along with elevated lipid peroxidation in the brain of rainbow trout (Li *et al*., 2010). Oxidative stress leads to mitochondrial dysfunction, accumulation of oxidized aggregated DNA, lipids and proteins with defects in protein clearance that finally constitute complex intertwined pathologies as severe neurological disorders and diseases (Halliwell, 2006). Oxidative stress in brain has been implicated in the progression of several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and many other neural disorders (Barnham *et al*., 2004; Uttara *et al*., 2009). Thus chlordecone is proved as neurotoxic to the fish, *Pseudetroplus maculatus* when exposed at sublethal concentrations for 96 h.

Further, it should be noted that the comparison of tissues in eradicating the free radical generation concludes that all tissues are equally affected due to the induction of chlordecone exposure. Among all tissues, gill showed primary defensive mechanism to prevent the toxicant induced free radical generation, but the attempt was failed. The fluctuation in the activities of antioxidant enzymes in different tissues may be due to specific adaptive mechanism adopted by the tissues in order to escape from the toxicant (Isik and Celik, 2008). However, it is suspected that chlordecone may prove more toxic to the exposed fish when treated chronically, because chlordecone when exposed only for 96 h showed irreversible tissue damages as histopathological alterations in gill, liver and brain tissues (refer chapter 7).

5.5 Effects of chlordecone on the activity of alkaline phosphatase (ALKP)

Alkaline phosphatase is a stress marker enzyme that serves as a diagnostic tool for detecting toxicity stress of chemicals in various tissues such as gill, liver and muscle. The enzyme is efficient in removing phosphate groups from many types of molecules such as, nucleotides, proteins and alkaloids (Dyhrman and Palanik, 1999). Alkaline phosphatase is a hydrolytic lysosomal enzyme released by the lysosomes for the hydrolysis of foreign material. Alkaline phosphatase is also involved in the mediation of membrane transport and transphosphorylation, which is found in all cell membranes where active transport occurs, therefore, is frequently used to measure the integrity of the plasma membrane (Akanji *et al.,* 1993). The present study showed concentration and time-dependent decrease in the activity of alkaline phosphatase in both gill and liver tissue when exposed to chlordecone. The inhibition might be probably due to the leakage of the enzyme from cytosol across the injured plasma membrane into the general blood circulation or decreased enzyme synthesis as a result of organ dysfunction, which could often lead to disturbed membrane transport and upset structural integrity of cells (Bakde and Poddar, 2011; Chitra and Maiby, 2014). Inhibition of alkaline phosphatase was also reported in the gill when exposed to quinalphos and also in the liver when exposed to silicon dioxide nanoparticles of the fish, *Oreochromis mossambicus* (Ramya *et al.,* 2015; Vidya and Chitra, 2015).

5.6 Effects of chlordecone on the activity of acetylcholinesterase (AChE)

Acetylcholinesterase activity is routinely employed as a biomarker of neurotoxicity when exposed to certain groups of contaminants, especially pesticides. It is a serine protease enzyme found in the cholinergic brain synopsis and neuromuscular junctions and mediates the hydrolysis of acetylcholine, a neurotransmitter (Quinn, 1987). The present study reported that chlordecone at both sublethal concentrations inhibited the activity of acetylcholinesterase in the brain in concentration and time-dependant manner than the corresponding control groups. Similar results has been reported in *Labeo rohita,* when exposed to methyl parathion, in which AChE activity was inhibited by 74% at 1.8mg/L and 90% at

5.4mg/L confirming the neurotoxic effects of the pesticide (Sivaperumal and Sankar, 2011). The decrease in the activity of AChE in the brain tissue has been reported when exposed to sublethal concentrations of bisphenol A, nonylphenol and fullerene after 96 h exposure in the fish *Pseudetroplus maculatus* (Rejitha *et al*., 2016; Asifa and Chitra, 2016b; Sumi and Chitra, 2017b). Likewise, a decrease in activity of AChE was reported in brain followed by muscle, gill, and liver of *Cyprinus carpio* when exposed to chlorpyrifos after 14 days at 0.0224 mg/L and 0.0112 mg/L concentrations (Halappa and David, 2009). Thus, exposure to contaminants, even at very low concentrations can inhibit AChE, which leads to an accumulation of acetylcholine at central cholinergic synapses and neuromuscular junctions (Varo *et al.*, 2003). Accordingly, the collapse in the nerves of brain could lead to behavioural modifications in fish. Such types of reduced AChE-linked unusual behaviours like hyperactivity, vertical positioning and loss of equilibrium were observed in *Clarias batrachus*, when exposed to a fungicide, Trizole (Propiconazole) (Srivastava and Singh, 2014). Similarly, chlorpyrifos at sublethal concentrations has been shown to cause reduction in the activity of acetylcholinesterase along with altered locomotor behaviours and accumulation of acetylcholine at synaptic junctions in mosquito fish (Rao *et al*., 2005). Chlordecone also induced behavioural modifications such as slow movement and lethargy, hitting on the side walls, reddening of eyes and fins as well as exophthalmia in *Pseudetroplus maculatus*, which was discussed in chapter 1. Thus the results of the present study clearly demonstrate that chlordecone is also neurotoxic to the cichlid fish *Pseudetroplus maculatus*, as evidenced by the alteration in the antioxidant enzymes and inhibition of the neurotransmitter enzyme, AChE in brain tissue even at sublethal concentrations exposed for 96 h.

In brief, the evidence presented in the present chapter along with the data from literatures, strongly established to a conclusion that chlordecone induced oxidative stress in the fish, *Pseudetroplus maculatus* and recommended as early-warning bioindicator of chlordecone pollution in the aquatic ecosystem.

Conclusions

1. Short-term exposure to chlordecone altered the antioxidant status in gill, liver and brain tissue of cichlid fish, *Pseudetroplus maculatus*.

2. Gill tissue though attempted an adaptive response to get rid of free radicals by increasing the activities of antioxidant enzymes failed in its defensive mechanism by the induction of lipid peroxidation.

3. Liver showed the imbalance of pro-oxidant and antioxidant system as evident by the 8-fold increase in the level of hydrogen peroxide that ultimately leads to the induction of lipid peroxidation.

4. Activities of all antioxidant enzymes in the brain were inhibited by 29 to 80% along with induction of hydrogen peroxide (13 to 20 times) and lipid peroxidation (6 to 11 times) thereby indicating the induction of oxidative stress.

5. Alteration in the tissue-specific stress marker enzyme, alkaline phosphatase in gill and liver tissues indicates organ dysfunction, which could often lead to disturbed membrane transport and upset structural integrity.

6. Change is the activity of acetylcholinesterase enzyme indicates the failure of nerve impulse transmission, which is confirmed by abnormal behaviour in the exposed fish.

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CHAPTER 6

Effects of chlordecone on metabolic enzyme activities

Introduction

1.1 Role of metabolizing enzymes in biotransformation

Metabolizing enzymes are diverse group of proteins that play an important role in metabolizing wide variety of xenobiotic chemicals such as industrial chemicals, drugs, pesticides, fertilizers, plastics and plastisizers, pollutants from sewage treatment plants, secondary metabolites and toxins produced by molds, plants and animals (Parkinson, 1996). Most of the xenobiotics are persistent in the environment for several years leading to bioaccumulation or biomagnification and enter into higher organisms indirectly through the food chain (Connell, 1989). The other mode of entry of xenobiotics into the body of organisms is by direct absorption through the skin, while some are absorbed by active transport mechanisms into lungs or gastrointestinal tract. When a xenobiotic enter into the body of an organism, it undergoes certain modifications called biotransformation, which is catalyzed by an array of enzymes in the liver and other tissues through which the lipophilic compound is transformed into a more water-soluble compound or as less toxic metabolites. As a result, the cell maintains chemical and functional homeostasis by changing the physical properties of the xenobiotics from lipophilicity, which favour rapid absorption; to hydrophilicity that favours easy excretion through urine, feces or through gills and also prevent from the re-absorption by kidney tubules (Timbrell and Marrs, 2009). Exceptionally volatile compounds are eliminated by exhalation rather than the general rule of biotransformation to nonvolatile, water-soluble chemicals, which relatively slows down the rate of elimination (Parkinson, 1996).

Generally in all organisms xenobiotics are metabolized by four different kinds of reactions namely oxidation, reduction, hydrolysis, and conjugation (Parkinson and Ogilvie, 2008). Phase I reactions include oxidation, reduction, and hydrolysis that are also known as functionalization, whereas the conjugation reactions are called Phase II reactions. Recently, a third phase of metabolism namely Phase III reactions or the antiporter system are recognized. It is an additional detoxification process which is highly concentrated in the tips of the villi of small intestine, which helps both in conjugation and elimination of toxins (Crettol *et al*., 2010).

1.2 Phase I reactions

The phase I reactions includes oxidation, reduction and hydrolysis of xenobiotics, resulting in either more hydrophilic molecules or more reactive molecules, which usually acts as substrates for phase II reactions. Phase I reactions usually convert the toxic parent xenobiotics to a more polar metabolite by either introduction or modification of functional groups such as hydroxy (-OH), amino ($-NH_2$), thiol (-SH) or carboxyl (-COOH) groups (Williams, 1971). Biotransformation of hydroxy (-OH) group occurs by oxidation, methylation, glucuronide conjugation or sulfate conjugation. Carboxyl (-COOH) group undergo biotransformation by oxidation, glucuronide conjugation and glycine conjugation whereas amino ($-NH_2$) group by deamination, methylation and glucuronide conjugation. Oxidation is the most common type of biotransformation and it involves side-chain hydroxylation, aromatic hydroxylation, dealkylation, dehydrogenations, sulphoxide formation or deamination of mono and diamines. Reduction reactions are relatively uncommon and it involves reduction of nitro, nitroso and azo groups. However, hydrolysis is a common biotransformation route for most of the esters and amides (Ionescu and Caira, 2005).

The process of biotransformation occurs though the primary and principal organ of first pass site of metabolism that varies among the species. However, the principal location for biotransformation reactions occurs in liver, although kidney, intestine, skeletal muscle, lungs, brain or even plasma may act as major sites of metabolism. Within the cells the metabolizing enzymes are found predominantly in endoplasmic reticulum, mitochondria, lysosome, cytosol, microsome and also in nuclear envelop and plasma membrane (Croom, 2012). The activities of the enzymes in the cell organelles is influenced by several factors as xenobiotics, hormones, stress, temperature, nutritional status of the animals and pathological conditions. Accordingly either an induction or reduction in the activities of enzymes occurs which are quantitatively measured by biochemical analysis. Major metabolic enzymes of oxidative phase I reaction includes cytochrome P450 enzymes, flavin containing monooxygenases, monoamine oxidases, xanthine or aldehyde oxidases.

1.2.1 Cytochrome P450 (CYP) enzyme system

Cytochrome P450 enzyme system is a large superfamily of hemoprotein mixed function oxidase that are responsible for the biotransformation of xenobiotics widely present in living species from bacteria to human. In the spectra of microsomal fraction, the protein absorbs the visible light at about 450 nm when reduced and subsequently form complex with carbon monoxide. Since the protein is hemoprotein where 'P' stands for pigment, the protein was later named as cytochrome P450 (Omura and Sato, 1964). It represents the most important Phase I biotransformation enzyme based on the catalytic versatility and on the activation and detoxification of number of xenobiotics (Guengerich, 1987; Waterman and Johnson, 1991). Nowadays, more than 300 cytochrome P450 isoforms and 11,500 genes have been identified where the substrates of P450 ranged from small of molecular weight 28kDa to large of molecular weight 1201kDa (Testa and Kramer, 2007). Based on the probable structural and functional similarities, cytochrome P450s are grouped into several families and subfamilies (Nelson *et al*., 1996). There are about 74 families have been reported but only about seventeen families are described in detail in human (Cupp and Tracy, 1998).

Studies have reported that most of the biotransformation reactions occurring are mediated primarily by enzymes of the CYP1, 2, 3 and 4 families, with CYP3A4 as the most copious isoform in humans (Shimada *et al*., 1994). Characterization of CYP genes in fish has been performed intensively over last several years. One of the studies have reported 137 genes encoding P450s and these genes are classified into 18 CYP families like CYP1, CYP2, CYP3, CYP4 etc. in Japanese pufferfish, *Takifugu rubripes* (Uno *et al*., 2012). Usually, cytochrome P450 enzymes are bound to either microsomal or to inner mitochondrial membranes. Mitochondrial CYPs are found in the steroidogenic organs, mainly concerned with steroid biosynthesis and are not involved in the metabolism of xenobiotics (Oinonen, 1996). However, most of the microsomal CYPs are generally involved in the biotransformation process of xenobiotics. It is present in almost all tissues, but highest concentration is found in endoplasmic reticulum (microsomes) of liver (Parkinson, 1996). Thus the hepatic microsomal P450 enzymes play a very crucial role in the detoxification of

xenobiotics and also play a key role in determining the intensity and duration of action of drugs. In addition, microsomal and mitochondrial P450 enzymes play key roles in the biosynthesis or catabolism of steroid hormones, fat-soluble vitamins, bile acids, eicosanoids and fatty acids that highlight the catalytic versatility of cytochrome P450 (Parkinson, 1996).

Phase I reactions are mainly catalyzed by the cytochrome P450 and flavin-containing monooxygenases. Oxidation reactions in Phase I pathway involves the incorporation of oxygen into the xenobiotic substrate (RH) which is catalysed by a group of microsomal membrane-bound mixed function oxidase located in the smooth endoplasmic reticulum of the liver and other extra-hepatic tissues (Gibson and Skett, 1994; Testa, 1995). The characteristic aspect of microsomal reaction is the reliance on electron transport chain enzyme such as nicotinamide adenine dinucleotide phosphate (NADPH) and the prerequisite for molecular oxygen (Jimenez and Stegeman, 1990). The general scheme of monooxygenase enzyme reaction is:

$$RH + NADPH + H^+ + O_2 \xrightarrow{\text{cytochrome P450}} ROH + NADP^+ + H_2O$$

Where RH is xenobiotic substrate and ROH is hydroxylated product.

1.2.1.1 Mechanism of xenobiotics activated CYP induction

Cytochrome P450 enzymes transform different types of environmental pollutants such as polycyclic aromatic hydrocarbons, heterocyclic amines, aromatic amines, nitrosamines and nitropolycyclic hydrocarbons. The xenobiotics usually first binds to the intracellular aryl hydrocarbon receptor (AhR), a multimeric complex consisting of a heat shock protein (hsp 90) and Ah receptor protein. The liganded AhR is then translocated into the nucleus and dimerise with AhR translocator protein, Aryl receptor nuclear translocase (ARNT), which is a competent DNA binding protein acts as a ligand-dependent transcription factor that binds particularly to the aryl hydrocarbon responsive element (AhRE) in the CYP1A1 gene and subsequently activates its expression (Hoffman *et al*., 1991; Whitlock *et al*., 1996; Bertilsson *et al*., 1998). Thus xenobiotics are transformed to

biologically reactive intermediate, usually electrophile such as epoxide, hydroxylamine and acyl halide (Gonzales, 2005). Even though these reactive intermediates are short-lived, they are able to become covalently attached to nucleophiles found in proteins, lipids, or DNA causing cell damage. Thus the scavenger systems such as glutathione conjugate the electrophiles and reduce the toxic effect of the reactive metabolite and finally eliminate either through urine or faeces (Furge and Guengerich, 2006).

Over the past two decades, the mechanism of CYP1A1 induction by xenobiotics has been most extensively studied among all the CYPs (Okey, 1990). Xenobiotics usually stimulate the expression of CYP1A1 gene resulting in the increased levels of messenger RNA that delivers the information to the cytosol for the synthesis of CYP1A1 protein. The induction or inhibition of CYP indicates that the pollutant disrupts the physiological equilibrium of the exposed fish, which lead to serious clinical consequences (Lin and Lu, 1998). Increased activities of CYP enzymes improves the potency of biotransformation system to metabolise the xenobiotics into more polar or hydrophilic excretable products or in some cases, this may lead to the formation of more toxic metabolites than the parent compound, which is able to damage DNA and other cellular macromolecules (Stegeman and Klopper-Sams, 1987; Goksoyr, 1995). At the same time, contaminant induced inhibition of CYP reactions can lead to increased toxicity and bioaccumulation factors in fishes and other animals (Lech and Bend, 1980).

Induction of CYP system by xenobiotic exposure can be measured by different levels such as levels of mRNA, protein and enzyme activity (Bucheli and Fent, 1995). The activity of biotransformation enzymes have been studied at the enzyme level with prototype substrate that undergo particular functional group transformations (Burke and Mayer, 1974). Induction of cytochrome P450-dependant monooxygenases, especially CYP1A protein by xenobiotic is associated with the increase in 7-ethoxyresorufin-*O*-deethylase (EROD) (Whyte *et al*., 2000). The activities of EROD along with aryl hydrocarbon hydroxylase (AHH) are the most sensitive biomarkers for determining the CYP induction responses in fish (Siroka and Drastichova, 2004).

1.2.2 Microsomal flavin-containing monooxygenase

Microsomal flavin-containing monooxygenase is the second most important monooxygenase system in the Phase I pathway of xenobiotic metabolism, especially in the metabolism of lipophilic amines (Rose and Castagnoli, 1983). The enzymes are predominantly flavoproteins containing a single molecule of FAD as the prosthetic group and are NADPH and oxygen-dependent (Ziegler, 1988). Flavin-containing monooxygenase enzyme system catalyses the oxygenation of nucleophilic organic compounds that contain nitrogen and sulphur involving in flavin reduction followed by oxygen binding (Testa, 1995). Then transfer of an internal electron to oxygen lead to the formation of peroxy-flavin complex. The substrate nucleophiles attack the distal oxygen of the hydroperoxide that results in the transfer of oxygen to the xenobiotic substrate (Ziegler, 1988). Unlike cytochrome P450, flavin-containing monooxygenase does not require the presence of xenobiotics to begin the catalytic cycle, but it possess notable 'cocked and loaded' mechanism to bind with NADPH and reduce FAD in the absence of substrate that are possibly under dietary and hormonal control (Daly, 1999). Apart from monooxygenation, it also catalyse oxidative decarboxylation, oxidative demethylation and disulfide bond formation (Gut and Conney, 1993).

Other enzymes that are involved in Phase I reactions includes peroxidases, dehydrogenases, monoamine oxidases, xanthine oxidases and aldehyde oxidases (Hodgson and Goldstein, 2001). Monoamine oxidase belongs to the family of flavin-containing amine oxidoreductases that are found abundant in outer membrane of mitochondria (Benedetti *et al*., 2006). Xanthine oxidase (XO) is a homodimer with 290kDa molecular mass that belongs to the molybdenum-protein family containing one molybdenum, one FAD and two iron-sulfurs. The enzyme catalyses the oxidation of hypoxanthine to xanthine and subsequently into uric acid, which in turn may be responsible for the higher level of XO (Cos *et al.*, 1998). The reduction of the enzyme results in the production of superoxide radical and hydrogen peroxide (Kelley *et al*., 2010). Aldehyde oxidase is a cytosolic enzyme of the molybdoflavo enzymes subfamily that catalyses the oxidation of aldehydes into carboxylic acids and also hydroxylation of some N-heterocycles (Garattini *et al*., 2008)

1.3 Phase II reactions

Phase II reactions are otherwise called conjugation reactions or synthetic reactions. It is the second step of biotransformation pathway that involves chemical combination of a compound with a conjugating agent namely carbohydrate, amino acids or compounds derived from them. Thus the conjugation reactions yield water-soluble, usually inactive and quickly excreted products which serve as detoxifying step in metabolism of drugs, xenobiotics or endogenous substrates. Sometimes conjugation reactions results in the formation of toxic metabolites such as reactive electrophiles thereby increase the toxicity of the parent compounds. Conjugation reactions usually involve activation of metabolite by forming high energy intermediate. Accordingly it is generally classified as type I and type II conjugation. Type I conjugation includes glucuronidation and sulfonation, in which an activated conjugating agent combines with substrate to yield the conjugated product. Type II conjugation include amino acid conjugation, in which the substrate is activated and then combined with an amino acid to yield a conjugated product such as glycine, taurine and glutamic acid (Hodgson, 2004). Apart from this Phase II biotransformation reactions also includes acetylation, methylation etc. thereby increase the hydrophilicity of xenobiotic and promote the excretion of foreign chemicals (Williams, 1959). Important Phase II enzymes are UDP-glucuronosyltransferase, glutathione-S-transferase, acetyltransferases and sulfotransferase.

1.3.1 UDP-glucuronosyltransferase (UGT)

UDP-glucuronosyltransferase is a cytosolic enzyme that catalyzes the transfer of the glucuronic acid component of UDP-glucuronic acid to a large number of endogenous and xenobiotic compounds (Phase I modified), including drugs, pesticides, carcinogens and other pollutants (King *et al.*, 2000). The resulting glucuronide is more polar and more easily excreted than the parent molecule. Moreover, the solubility of the product is increased in the blood allowing it to be eliminated from the body by the kidneys. UGT enzyme is devoid of prosthetic group and its catalytic activity is significantly influenced by the presence of lipids (Gibson

and Skett, 1994). It is also predominantly seen in the microsomal fractions of liver endoplasmic reticulum, but also in kidney, small intestine, lung, skin, adrenals and spleen. UDP-glucuronyltransferase mainly concerned with the glucuronidation of nucleophilic compounds (George, 1994). Multiple forms of UGT have been reported in human, rabbit and rat with broad substrate specificities (Tephly, 1990). Fish also possess multiple UGT isoforms with similar structural and functional properties to mammalian UGTs (Clarke *et al*., 1992).

1.3.2 Glutathione-S-transferase (GST)

The glutathione-S-transferase is a multifunctional, multigene superfamily of dimeric proteins (George, 1994). It is mainly found in the hepatic cytosolic and microsomal fractions, which catalyses the conjugation of reduced glutathione (GSH) with a wide variety of electrophilic xenobiotics and other endogenous compounds, which include carcinogens as well as various compounds that are the products of oxidative stress including oxidised DNA and lipid (Hayes and Strange, 1995). The activity of GSTs has been broadly characterized in human, rats and mice and it plays a pivotal role in protecting the cells from the consequences of oxidative stress (Pickett and Lu, 1989). Based on the protein sequence, substrate specificity and immunological activity, GSTs have been classified into different forms such as Alpha, Mu, Pi and Theta classes that are mainly located in the cytosol (Mannervik *et al*., 1985; Meyer *et al*., 1991). Different isozymes of GST were reported in both teleost and elasmobranch fishes (Nimmo, 1987). Characterization of GSTs in zebrafish demonstrates different types of GSTs such as Gstp1, Gstp2, Gstt1a, Gstz1, Gstr1, Mgst3a and Mgst3b, which play an important role in the biotransformation of xenobiotics. On the other hand, GST Alpha, Mu, Pi, Zeta and Rho classes are involved in the crucial physiological processes (Glisic *et al*., 2015). In addition to the role in detoxification and excretion of xenobiotics, GSTs are also concerned with the protection of cells from peroxidative damages and participates in the transport of endogenous compounds such as steroids, bilirubin and heme (Smith *et al*., 1989; Armstrong, 1991).

1.3.3 Sulphotransferase (SULT)

Sulphotransferase enzymes are important Phase II enzyme that catalyze the sulfation of a wide variety of xenobiotics and their metabolites, as well as numerous endogenous hormones, neurotransmitters, bile acids, carbohydrates and proteins forming highly water soluble sulfuric acid ester (Duffel, 2001). Sulfate conjugation usually involves the transfer of sulfonate functional group (SO_3^-) to the xenobiotic compound. The sulphotransferases are cytosolic soluble enzymes found in many tissues including liver, kidney, gut and platelets where they exist in multiple enzyme forms (Weinshilboum, 1990). There are four sulfotransferase gene families have been identified namely SULT1, SULT2, SULT4, and SULT6 and were purified and characterized in humans (Stanley, 2017). SULT was also purified and characterized from the hepatic and intestinal cytosol of the channel catfish (Tong and James, 2000).

1.3.4 Acetyltransferase

Acetyltransferases are cytosolic enzymes found abundant in liver and also in many other tissues like kidney, intestine and blood, which is responsible for the acetylation of xenobiotics with aromatic amine ($R–NH_2$) or a hydrazine group to yield more hydrophilic product (Evans, 1992). Humans, rabbits and hamsters express two isoforms namely NAT1 and NAT 2, whereas mice express three forms of acetyltransferase (Vatsis *et al*., 1995).

1.4 Phase III reactions

After the completion of Phase I and Phase II pathways, the unwanted, hydrophilic products of xenobiotic metabolism are now ready for excretion from the cell. The metabolites must be transported out of the cell in order to eliminate from the body, and it is accomplished by certain proteins called Phase III transporters or antiporter system. It carries the metabolites towards the blood circulation and finally to the kidneys. More than 350 unique antiporter proteins have been identified with the best known Phase III transporters are P-glycoprotein (Pgp) and multidrug resistance proteins (MRP) (Doring and Petzinger, 2014). Phase III reactions

decrease the intracellular concentration of xenobiotics thereby reduces toxic load in the liver. Thus the conjugated xenobiotics of Phase II reactions are pumped into the bile and intestinal lumen by the antiporter system so that it can be readily excreted from the body of the animal. But comparatively, the induction of biotransformation enzymes is less effective in fish than mammals. Fish has relatively low ability to metabolize xenobiotics because it is well adapted with excretory system in gill tissues that immediately eliminate xenobiotics to some extent as fish is directly in contact with an environment pollutant. However, studies have reported that the lower metabolic enzyme activities itself is adequate for the elimination of xenobiotics that the fishes usually encounter (Chambers and Yarbrough, 1976).

The present chapter is therefore focused to discuss the effects of chlordecone on the activities of different metabolic enzymes such as 7-ethoxyresorufin-*O*-deethylase, UDP-glucuronosyltransferase, glutathione-S-transferase and gamma glutamyltranspeptidase in the liver of *Pseudetroplus maculatus* at two sublethal concentrations in order to assess the mode of biotransformation of the toxicant.

Review of Literature

Pesticides pollution severely affects aquatic ecosystem and its effects on fishes are of great concern. Adverse effects of toxicants on the ecosystem and the extent of deleterious effects on natural resources and organisms exposed directly or the indirect effects to humans through food chain are analyzed by the ecological or environmental risk assessment. The ability of various toxicants and its derivatives to establish the potential harmful effects on population depends solely on the environmental levels of the toxicant (Dawe, 1990; Murchelano, 1990). Usually, an organism can eliminate the toxicant in two major ways such as excretion of the parent compound in its original form or by xenobiotic biotransformation (Chambers and Yarbrough, 1976). The hydrophilic toxicants do not require xenobiotic transformation because those chemicals are frequently degraded by abiotic processes like hydrolysis or photolysis. However, lipophilic xenobiotics are converted to more water soluble compounds through Phase I and Phase II biotransformation reactions, which is indispensable for the detoxification and excretion processes (Sijm and Opperhuizen, 1989). Phase I and Phase II biotransformation reactions are of great importance in the understanding of metabolism of endogenous molecules and transformation of xenobiotics and drugs, in fish and other animals (Gonzales *et al*., 2009). Moreover, certain steps in the biotransformation process are responsible for the activation of xenobiotics to reactive intermediates that finally result in toxicity, tumorigenicity and other adverse toxic effects (Varanasi, 1989). Thus, during biotransformation process, xenobiotics induce the formation of several free radicals and other reactive oxygen species, which has been known to cause several cellular damages, physiological alterations and carcinogenesis (Goeptar *et al*., 1995).

Fish eliminate a foreign compound by excreting as such through gills or it may convert to hydrophilic metabolite by biotransformation pathway, which is the major biochemical factor influencing the distribution and retention of toxic chemicals in fish (Chambers and Yarbrough, 1976). Similar to mammals, fishes are also capable of xenobiotic metabolism by both microsomal oxidation, reduction and conjugation reactions. The xenobiotic transforming enzymes appear to be similar between fishes and mammals, with the exception of lower temperature optima in fishes which may reveal the usual lower operating temperatures of heterotherms as

compared to homeotherms (Chambers and Yarbrough, 1976). In fish, the biotransformation enzymes are primarily distributed in the liver along with some extra-hepatic structures such as gut, kidneys, gills and the olfactory system (Chambers and Yarbrough, 1976; Kleinow *et al*., 1987). Unlike to humans, gills and the olfactory tissues of fish constitute direct target sites for waterborne pollutants, which are steadily in permanent contact with the external environment (Klaprat *et al*., 1992). The substantial biotransformation ability of the olfactory tissue in coho salmon in detoxification or biotransformation of waterborne pollutants has been identified by the expression pattern of various cytochrone P450 isoforms (Matsuo *et al*., 2008).

Cytochrome P450 system is one of the best studied biochemical markers for xenobiotic exposure in fishes (Goksoyr, 1995). Among different families of CYPs, CYP1A induction has been extensively studied and characterized in fish (Hahn and Chandran, 1996). The CYP system in fish demonstrate structural similarity to mammalian system, but comparatively the enzyme activity is lower in fish than the mammals, and are predominantly controlled by exogenous factors (Klotz *et al*.,1984; Stegeman and Hahn, 1994). The activity of cytochrome P450 enzyme system has been known to exhibit individual variations among the strain, concentration, activity and susceptibility within the same species. It could be due to the disparities in habitats, physiology, behaviour and feeding habitats of the animal in addition to the differences in uptake, accumulation, distribution and metabolism of contaminants exposed (Goksoyr and Forlin, 1992). Numerous CYP isoforms has been purified and characterized from both freshwater and marine fishes and widely studied CYP subfamilies include CYP1A, CYP2B/E/K, CYP3A, CYP4A, CYP11A, CYP17 and CYP19 (Stegeman and Hahn, 1994). The activity of CYP1A1 isozyme has been used frequently as a biomarker of aquatic pollution involving poly aromatic hydrocarbons and planar chlorinated hydrocarbons in the liver of fishes (Levine *et al*., 1994; Levine and Oris, 1999). Similarly, one of the CYP1A catalyzed enzyme, 7-ethoxyresorufin-*O*-deethylase constitutes another important biomarker of aquatic pollution in fish. Activities of cytochrome P-450-dependent monooxygenases such as EROD, aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase has

been reported to be increased in the hepatic microsomes of nase (*Chondrostoma nasus*), roach (*Rutilus rutilus*) and grayling (*Thymallus thymallus*) captured from polluted areas of the river Rhone, France (Monod *et al*., 1988). One of the environmental contaminants, tetrachlorodibenzodioxin caused a rapid induction of CYP1A mRNA, followed by an increase in the concentration of CYP1A protein and EROD activity in the hepatocytes of rainbow trout *in vitro* (Lorenzen and Okey, 1990). Xenobiotic-induced EROD activities has been investigated in different species of fish throughout the world (Fent and Batscher, 2000; Aarab *et al*., 2004; Wafo *et al*., 2005; Ferreira *et al*., 2006). Exposure to 17alpha-ethynylestradiol has been shown to induce EROD activity about 8 to 9 times in the gill of three spined stickleback (Andersson *et al*., 2007). Induction of EROD has been reported in the stickleback fish sampled from French rivers characterized by various industrial, urban and agricultural contaminations (Sanchez *et al*., 2007). Similarly, Mullet caught from different locations of the Aliaga Bay contaminated with polycyclic aromatic hydrocarbon type organic pollutants showed induction of CYP activity, which is evidenced by 52 times more EROD activity than the control fish sampled from a clean reference site (Sen *et al*., 2010). Induction of EROD activity by the exposure to toxicants reinforce the induction of CYP1A in the fish and it substantiate that the biotransformation mechanism of the toxicant is mediated through Phase I reaction.

Sometimes the toxicity of the compound is highly reduced when the level of excretion is generally elevated. In several species of fishes, glucuronidation has been demonstrated as one of the most common pathways for the metabolism of several xenobiotics. Exposure to β-naphthoflavone has been shown to induce the induction of UDP-glucuronosyltransferase (UGT) in the liver of fish (Zhang *et al*., 1990; Clarke *et al*., 1991). Long-term exposure to polychlorinated biphenyles induced UGT, glutathione S-transferase and glutathione reductase activities in the liver of rainbow trout (Blom and Forlin, 1997). Whereas, nonylphenol and nonylphenol diethoxylate inhibited the activity of hepatic UGT in Atlantic salmon and rainbow trout (Arukwe *et al*., 1997; Sturm *et al*., 2001). Treatment of juvenile salmon with nonylphenol caused decreased activities of 7-ethoxyresorufin *O*-deethylase, UDP-

glucuronosyltransferase, CYP1A, CYP2K and CYP3A in hepatic microsomes along with decrease in progesterone 6β-, 16α-, and 17α-hydroxylase activities (Arukwe *et al*., 1997). Sea bass when exposed to *β*-naphthoflavone and benzopyrene has been shown to induce hepatic EROD activity along with induction of erythrocytic nuclear abnormalities (Gravato and Santos, 2002). Organoselenide exposure has been reported to cause induction of flavin monooxgenase in shark indicating the involvement of flavin monooxygenase in the oxidation and initiation of the contaminant metabolism (Schlenk *et al*., 2003).

One of the polycyclic aromatic hydrocarbon, phenanthrene induced a concentration-dependant formation of the 7-ethoxyresorufin-*O*-deethylase in the liver along with elevated activity of sorbitol dehydrogenase indicating the contaminant induced liver cell damages in *Oreochromis mossambicus* (Shailaja and D'Silva, 2003). Exposure of tributyltin to juvenile salmon for seven days has been shown to induce the expression of CYP3A and UGT mRNA signifying the involvement of CYP3A and UGT in the metabolism of the contaminant in fish (Mortensen and Arukwe, 2007). Exposure to hydroxy-polychlorinated biphenyl has been found to decrease the expression of CYP1A1, UGT and GST in primary culture of Atlantic salmon hepatocytes (Mortensen *et al*., 2007). Induced activities of EROD and UGT in African sharptooth catfish caught from sewage ponds in Tanzania revealed that all sewage ponds were contaminated by pollutants of public health concern (Mdegela *et al*., 2010). Primary hepatocyte cell cultures of Atlantic cod exposed to β-naphthoflavone, cadmium and polychlorinated dibenzo-*p*-dioxin showed elevated expression of CYP1A mRNA and down-regulation of UGT mRNA (Softeland *et al*., 2010). In a study involving the evaluation of microsomal biotransformation of chlorpyrifos, parathion and fenthion in the gills, liver and olfactory tissues of rainbow trout and coho salmon, has been found that bioactivation and detoxification mechanisms of these organochlorine pesticides were more in liver than in other tissues and rainbow trout is more sensitive than coho salmon to the acute toxicity of the pesticides because trout have higher microsomal catalytic activity (Lavado and Schlenk, 2011). Gemfibrozil, human lipid regulator, has been shown to inhibit CYP1A, CYP3A and CYP2K-like catalytic

activities without altering the Phase II enzymes as UDP-glucuronyltransferase and glutathione-*S*-transferase in European eel (Lyssimachou *et al*., 2014). Detoxification responses of emamectin benzoate, one of the drugs used for the treatment and control of infections by *Caligus rogercresseyi* on salmonids have been evaluated in rainbow trout. The results of the study reveal that the drug impairs the detoxification processes and immuno-modulatory mechanisms, which is evidenced by the alterations in the expression and activity of CYP1A, FMO and GST (Carcamo *et al*., 2014).

Mixture of benzopyrene and chrysene when exposed to *Chlamys farreri* have been shown to induce EROD, UGT and sulfotransferase, along with induced expressions of aryl hydrocarbon receptor, CYP1A1, CYP1B1, multidrug resistance protein and breast cancer resistance protein, however, the activity of glutathione-S-transferase was inhibited. The chemical mixture also caused induction of oxidative stress demonstrating the toxic potential of the contaminant mixture in the exposed animal (Guo *et al*., 2017). Two estrogenic contaminants, benzophenone-2 and bisphenol-S treated to zebrafish embryos and adults reported that biotransformation pathway of bisphenol-S was chiefly through glucuronidation in both adults and larvae, whereas benzophenone-2 exerted biotransformation through glucuronidation as the major pathway in larvae and by sulfation pathway in adults (Le Fol *et al*., 2017).

Thus alterations in the activities of xenobiotic biotransformation enzymes have been used as one of the important biomarkers of environmental pollution in aquatic toxicology. Modifications in the activities of the biotransformation enzymes are also linked with normal physiological functioning in organisms. In the light of the above reviewed literatures, the present chapter was designed to evaluate the effects of chlordecone on the metabolic enzyme activities as the promising tool for the ecological risk assessment. However, the effects of the toxicant may vary based on the differences in species, age, sex, nutrition, pathological conditions and genetic differences in the exposed animal.

Materials and Methods

3.1 Chemicals

Triton X-100, 7-ethoxyresorufin and trichloroacetic acid were obtained from Sigma-Aldrich, USA. NADPH, reduced glutathione, *p*-nitrophenol, 1-chloro-2,4-dinitrobenzene, phenylmethylsulfonyl fluoride and glycylglycine were obtained from Himedia Laboratories, Mumbai, India and L-γ-glutamyl-3-carboxy-4-nitroanilide was purchased from Carbosynth Limited, UK. All other chemicals were of analytical grade and obtained from local commercial sources.

3.2 Experimental treatment

Sublethal concentrations of chlordecone i.e., one-tenth (3.5µg/ L) one-fifth (7µg/ L) of LC_{50}-96 h was exposed for 24, 72 and 96 h durations along with negative (without solvent-DMSO) and positive (1% DMSO-vehicle) controls. At the end of all treatment period, by using small dip net each fish was caught very gently without giving stress to the animal. Fish was killed by decapitation and liver tissue was dissected out and processed immediately for biochemical analysis.

3.3 Preparation of hepatic cytosolic and microsomal fractions

Hepatic cytosolic and microsomal fractions were prepared as described by Bradford, 1976. Briefly, after weighing, liver was chopped finely and 20% (w/v) homogenate was prepared in ice-cold 0.25 M sucrose with a Teflon homogenizer on crushed ice for a minute. Homogenate was centrifuged at 500*g* for 15 min at 4ºC, the fatty layer was removed and the supernatant obtained was centrifuged at 12,000*g* for 20 min at 4ºC. The obtained supernatant was further centrifuged at 100,000*g* for 60 min at 4ºC in Beckman Ultracentrifuge to obtain the cytosolic fractions. The microsomal pellets thus obtained were resuspended in 0.25M sucrose to get the microsomal fractions. Both cytosolic and microsomal fractions were stored at -80ºC until the biochemical analysis was performed. Protein was estimated by the method of Lowry *et al*. (1951) in both microsomal and cytosolic fractions of liver with BSA as the standard.

3.3.1 Assay of EROD (7-ethoxyresorufin *O*-deethylase)

EROD activity was determined in the microsomal fraction of the liver as described by Fernandes *et al*., 2002. Hepatic microsomes (200 µL) obtained from differential centrifugation was incubated at 30ºC for 10 min in 100 mM phosphate buffer, pH 7.4, 0.25 mM NADPH and 4.15 µM 7-ethoxyresorufin. The reaction was stopped by adding 2 mL of ice-cold acetone and absorbance was read at 537 nm. The standard curve was plotted using 7-hydroxyresorufin and the activity was expressed as the amount of resorufin generated/ minute/ mg protein.

3.3.2 Assay of UDP-glucuronosyltransferase (UGT)

UDP-glucuronosyltransferase activity was assayed according to the method of Zhivkov, 1970. Hepatic microsomal fraction was pretreated with 0.2% Triton X-100 on ice and the reaction was initiated by the addition of 81 µM *p*-nitrophenol in shaking water bath for 30 min at 30ºC. The reaction was stopped by the addition of 0.2 M ice-cold trichloroacetic acid, centrifuged at 800 *g* for 10 min and alkalinized with 0.1 mL of 10 N KOH and the optical density was measured at 405 nm. The enzyme activity was expressed as the amount of *p*-nitrophenol generated/ minute/ mg protein.

3.3.3 Assay of glutathione S-transferase (GST)

Glutathione S-transferase activity was assayed by the method as described by Habig *et al*., 1974. Briefly, the reaction mixture contained potassium phosphate buffer (80 mM; pH 7.4), 1-chloro-2,4-dinitrobenzene (1mM) and reduced glutathione (1 mM) were incubated with 100 µL of hepatic cytosolic fraction at room temperature. The change in absorbance was recorded at 340 nm and the enzyme activity was expressed as µM 1-chloro-2,4-dinitrobenzene conjugate formed/ min/ mg protein.

3.3.4 Assay of gamma-glutamyl transpeptidase (γ-GTP)

γ-GTP was measured by the modified method of Christiansen *et al*., 1998. Hepatic microsomal fraction was mixed with sodium hydroxide (60 mM, pH 7.9),

glycylglycine (150 mM) and L-γ-glutamyl-3-carboxy-4-nitroanilide (6 mM). The change in absorbance was measured at 410 nm for 5 min using Shimadzu UV-Visible spectrophotometer and the enzyme activity was expressed as nmol *p*-nitroaniline liberated/ min/ mg protein.

3.4 Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Post Hoc test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against the control groups. Data are presented as mean±SD for ten animals per group. All biochemical estimations were carried out in duplicate.

Results

4.1 Effects of chlordecone on the activities of metabolic enzymes

Chlordecone exposed at both sublethal concentrations significantly ($P<0.05$) increased the activities of ethoxyresorufin-*O*-deethylase and UDP-glucuronosyltransferase in concentration-dependant manner in the hepatic microsomal fractions when compared to the control groups (Figures 1 and 2). Chlordecone administration significantly ($P<0.05$) decreased the activity of glutathione-S-transferase in concentration and time-dependant manner in the cytosolic fractions of liver when compared to the corresponding control groups (Figure 3). The activity of gamma-glutamyl transpeptidase increased significantly ($P<0.05$) in the microsomal fractions of liver at both sublethal concentrations in chlordecone treatment (Figure 4). Exposure to vehicle control (1% DMSO) showed no remarkable changes in the activities of all enzymes and the activities were found similar to the solvent-free (negative) control group.

Figure 1: **Effect of chlordecone on the activity of 7-ethoxyresorufin-*O*-deethylase in hepatic microsomal fractions in the fish, *Pseudetroplus maculatus***

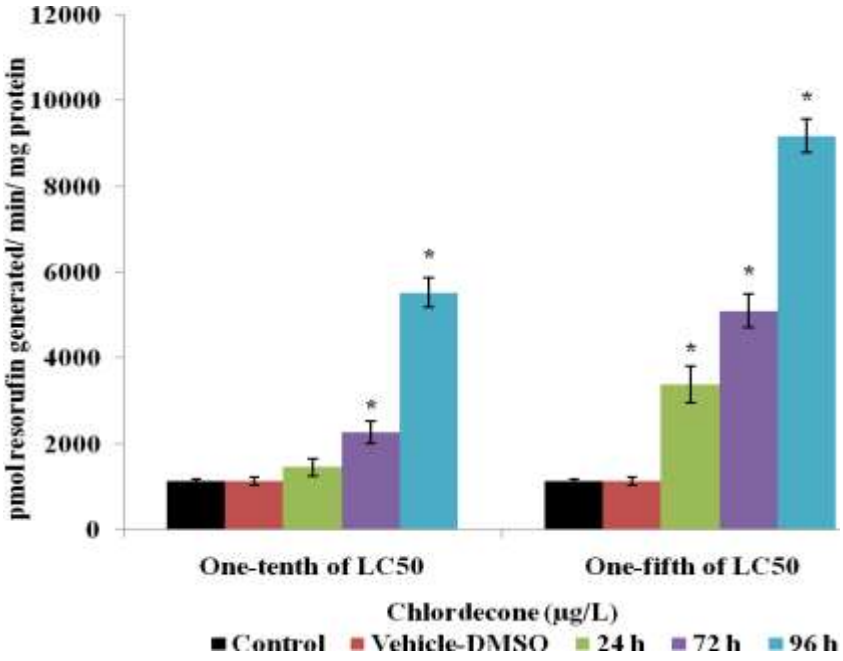


Figure 2: **Effect of chlordecone on the activity of UDP-glucuronosyltransferase in hepatic microsomal fractions in the fish, *Pseudetroplus maculatus***

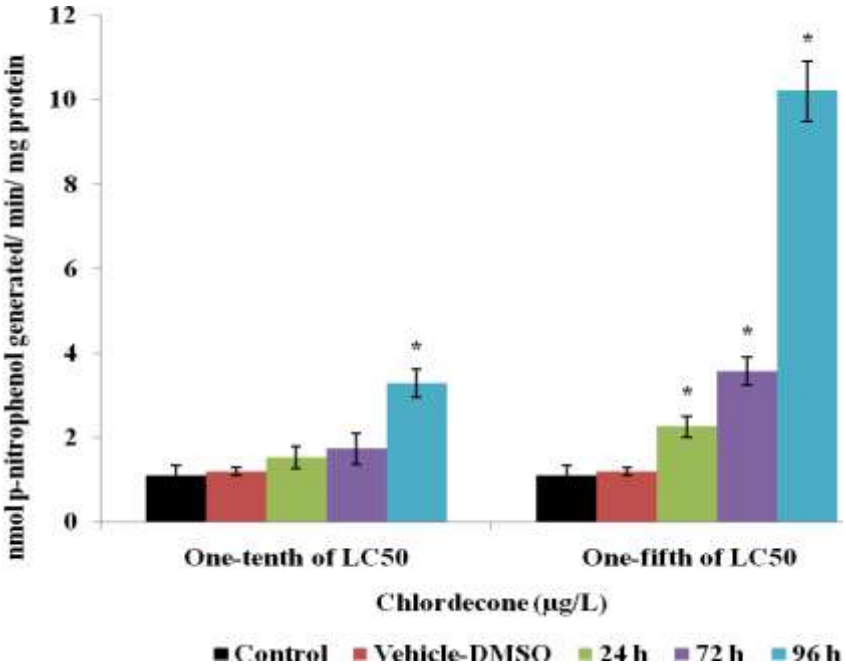


Figure 3: **Effect of chlordecone on the activity of glutathione-S-transferase in hepatic cytosolic fractions in the fish, *Pseudetroplus maculatus***

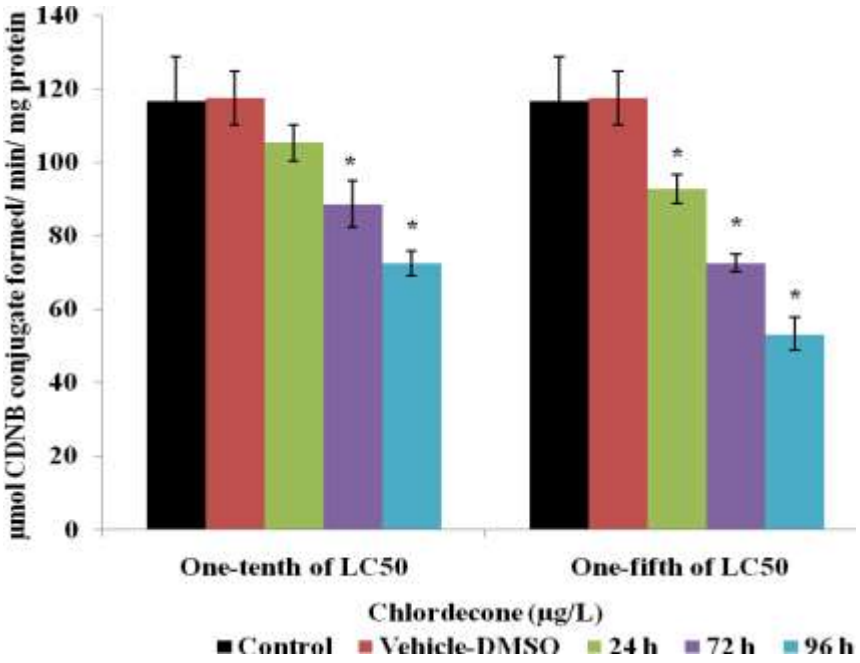
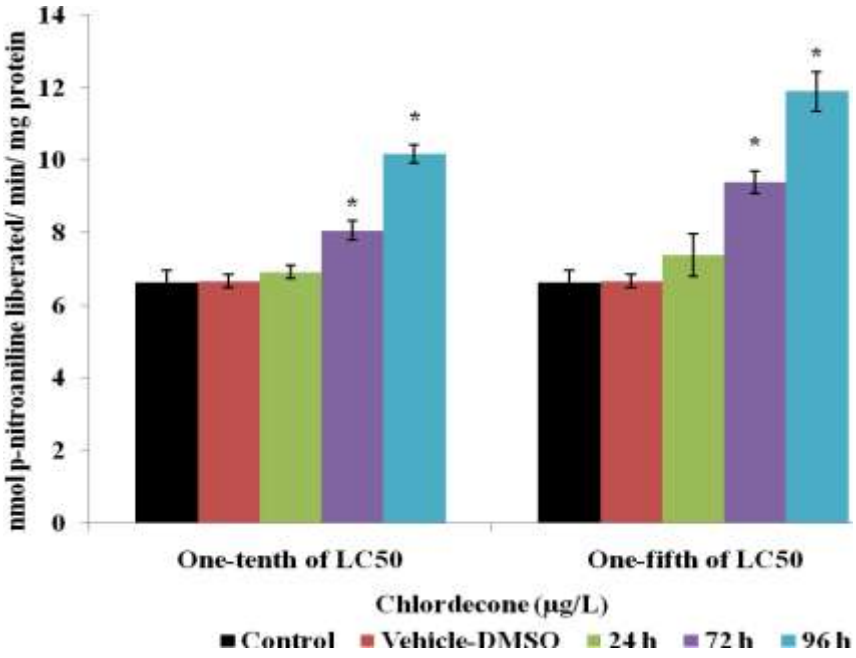


Figure 4: **Effect of chlordecone on the activity of gamma-glutamyl transpeptidase in hepatic microsomal fractions in the fish, *Pseudetroplus maculatus***



Discussion

5.1 Effect of chlordecone on the activity of 7-ethoxyresorufin-*O*-deethylase

The present study evaluated chlordecone induced metabolic activity of Phase I reaction enzyme namely 7-ethoxyresorufin-*O*-deethylase (EROD). EROD is an important biomarker enzyme frequently used to measure the activity of cytochrome P450-dependant monooxygenase induction due to the exposure of xenobiotic chemicals (Stegeman *et al*., 1997). The CYP1A subfamily of cytochrome P450 system in fish is mainly responsible for the biotransformation of a broad range of xenobiotics such as polycyclic aromatic hydrocarbons (Benedetti *et al*., 2007). The enzymatic assay of EROD is usually conducted in hepatocytes of fish because liver is the tissue mainly concerned with highest rate of xenobiotic metabolism (Payne *et al*., 1987). The catalytic activity of EROD is represented by the rate of CYP1A-mediated deethylation of the substrate, 7-ethoxyresorufin, to form the product resorufin (Kennedy and Jones, 1994).

Induction in the activities of ethoxyresorufin- and ethoxycoumarin-*O*-deethylases and the content of cytochrome P450 has been reported when chlordecone was administered at 45 mg/kg weight in mice (Carpenter and Curtis, 1991). In another study, chlordecone at different concentrations induced P4501A and 2B responses and declined the level of reduced glutathione in human hepatocellular carcinoma cell lines (Dehn *et al*., 2005). Generally, the inducers of CYP1A and EROD are agonists of aryl hydrocarbon receptor, especially organic contaminants belonging to chlorinated pesticides, polychlorinated biphenyls, dioxins and aromatic hydrocarbon (Mikula *et al*., 2009; Dong *et al*., 2009; Haluzova *et al*., 2011). EROD activity and cytochrome P4501A were also induced in the liver and gills of Senegal sole sampled from polluted regions of Huelva estuary, Spain (Oliva *et al*., 2014). In the present study, chlordecone administration caused significant increase in the activity of 7-ethoxyresorufin-*O*-deethylase in the hepatic microsomal fractions of *Pseudetroplus maculatus* at both sublethal concentrations in time-dependant manner. Thus the present result indicates that chlordecone has the ability to bind and activate aryl hydrocarbon receptor and function as competitive inhibitor for CYP1A1 in the fish.

5.2 Effect of chlordecone on the activity of UDP-glucuronosyltransferase

Phase II reactions of biotransformation pathway convert the endogenous xenobiotic compounds into more easily excretable and metabolically inactive form by a series of enzymes. Glucuronidation is a key pathway for the metabolism and excretion of both endogenous and exogenous compounds in fishes and other vertebrates (Dutton, 1980). The key enzyme involved in the process of glucuronidation is UDP-glucuronosyltransferases (UGT) enzymes that are responsible for the metabolism of many xenobiotics. In all vertebrates including fish, the formation of glucuronide conjugates is the most important detoxifying pathway of the Phase II reactions. In the present study, chlordecone exposure increased the activity of UDP-glucuronosyltransferase at both concentrations and about 9-fold increase was observed at 7μg/L concentration after 96 h of exposure. The present observation clearly demonstrates that the polar metabolite of chlordecone formed in Phase I reaction enter into glucuronide conjugation reaction and are either ready to get eliminated from the body or is biotransformed into intermediate compounds. The formation of glucuronide conjugates after 96 h exposure of chlordecone is evident by the significant ($P<0.05$) increase in the activity of UDP-glucuronosyltransferase in the microsomal fractions of fish liver.

The catalytic domain of the integral microsomal glycoprotein enzyme, UGT, is oriented towards the lumen of endoplasmic reticulum. The destruction of vesicular structure of microsomes as a result of chlordecone exposure could have routed UDP-glucuronic acid to the active site of the enzyme through the hole in the microsomal membrane. This could be the reason for the increase in the activity of UGT enzyme in hepatic microsomal fractions of chlordecone-treated fish. However, the present study also revealed that 100% of chlordecone is not biotransformed to chlordecone metabolites as the toxic adverse effects of chlordecone is noted in both reproductive and non-reproductive tissues that was discussed in the previous chapters. It has been reported that certain biotransformation processes are responsible for the formation of intermediate compounds that are more toxic than the parent compound (Guengerich, 1992) and this could be one of the reasons behind the toxicity of chlordecone in the exposed fish.

5.3 Effect of chlordecone on the activity of glutathione S-transferase

Glutathione S-transferases (GST) are one of the most studied enzymes of Phase II reactions, which are involved in the detoxification of various xenobiotics that catalysis the conjugation of reduced glutathione with compounds containing electrophilic functional groups (Riol *et al*., 2001). Glutathione S-transferase has been shown to mediate cellular defences against toxic and reactive electrophiles such as reactive oxygen species, which are formed as a result of normal metabolic processes or exposure to various environmental contaminants (Jancova *et al*., 2010). In the present study, chlordecone decreased the activity of glutathione S-transferase in concentration and time-dependant manner in the cytosolic fractions of liver. The inhibited activity of glutathione S-transferase after chlordecone exposure could be due to the failure of the enzyme to protect the hepatocytes against the toxic exposure (Taya *et al*., 2014). Both GST and glutathione (GSH) are involved in the protection of liver against a number of toxic compounds and the decrease in the activity of GST suggests the active circulation of chlordecone, which in turn is responsible for the increase in the hepatotoxic action of the exposed toxicant. The present result coincides with other studies where exposure to deltamethrin caused decrease in the activity of GST in the gill of *Channa punctatus* and in the gill and liver of *Ancistrus multispinis*, respectively (Solomon *et al*., 2000; Pimpao *et al*., 2007).

5.4 Effect of chlordecone on the activity of γ-glutamyl transpeptidase

Gamma-glutamyl transpeptidase (γ-GTP) is an enzyme involved in the homeostasis of glutathione and it also initiates metabolism of glutathione-S-conjugates to mercapturic acids by catalysing gamma-glutamyl moiety to a variety of acceptor molecules including water, certain amino acids and peptides thereby leaving the cysteine product (Zhang *et al*., 2005). In addition, γ-GTP is also a specific marker enzyme of Sertoli cells and in cell proliferation action of tumour cells. γ-GTP is concentrated in the liver, and also detectable in biliary epithelium, occasionally in periportal hepatocytes, gallbladder, spleen, pancreas and kidneys (Lindros *et al*., 1989). The present study showed that chlordecone at both sublethal concentrations increased the activity of gamma-glutamyl transpeptidase in the

hepatic microsomal fractions of *P. maculatus*. The elevated level of gamma-glutamyl transpeptidase is associated with lipid peroxidation in liver cells (Paolicchi *et al*., 1997) and the induction of lipid peroxidation by chlordecone exposure was already discussed in chapter 5. The mechanism behind γ-GTP-dependent lipid peroxidation could be due to the higher production of cysteinyl-glycine that possesses the ability to induce lipid peroxidation through the interactions with iron (Paolicchi *et al*., 1997). Thus the increase in the activity of γ-GTP in hepatocyte is directly associated to the induction of lipid peroxidation after chlordecone exposure in *Pseudetroplus maculatus*.

Metabolism of chlordecone discussed in the present chapter clearly indicates that chlordecone is metabolized through both Phase I and Phase II reactions. There are earlier report stating that the metabolism of chlordecone to chlordecone alcohol is known to occur predominantly in the liver and only 20-40% eliminated exclusively though faeces (Houston *et al*., 1981). Chlordecone alcohol, the metabolite of chlordecone, found in bile is predominantly in the form of glucuronide conjugate (93%). Among 20% of chlordecone elimination, 72% are primarily excreted as an unaltered parent compound and the remaining as conjugated-form along with glucuronic acid. The bioreduction of chlordecone to chlordecone alcohol is catalyzed by a hepatic cytosolic enzyme called chlordecone reductase (Molowa *et al*., 1986). Thus it is suggested that the major metabolic route for chlordecone is by its reduction in the liver through Phase I reaction followed by glucuronidation, which is through conjugation process of Phase II reaction (Fariss *et al*., 1980). The present study was also in agreement to the literatures reviewed that chlordecone biotransformation occurs primarily by inhibiting CYP1A1 (Houston *et al*., 1981). It also undergo type I conjugation of Phase II reaction, including glucuronidation and sulfonation, as evident by the alteration of UDP-glucuronosyltransferase and glutathione-S-transferase activities.

Conclusions

1. Chlordecone augments with aryl hydrocarbon receptor and function as competitive inhibitor for CYP1A1 in the fish through Phase I reaction, which is evidenced by the increase in EROD activity.

2. Chlordecone biotransformation occurs by type I conjugation of Phase II reaction such as, glucuronidation and sulfonation, which is evidenced by alteration in the activities of UGT and GST.

3. However, the reactive end products of chlordecone metabolism resulted in toxicity, which is evidenced by the altered activities of metabolic enzymes in hepatocytes.

4. Thus metabolism of chlordecone occurs through both Phase I and Phase II reactions, where the fish has attempted to detoxify the substrate or its metabolites in the liver, but failed and resulted in toxicity in the fish, *Pseudetroplus maculatus*.

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CHAPTER 7

Effects of chlordecone on histopathology of reproductive and non-reproductive tissues

Introduction

1.1 Histopathology as biomarker of toxicant exposure

Histopathology entails the microscopic examination of cells and tissues of an organism to study the manifestations of xenobiotic contamination and several diseases in fishes and other animals (Bernet *et al*., 1999; Gupta *et al*., 2009). It is a highly sensitive, valuable and easiest tool to assess the toxic effects of environmental contaminants in fish. Therefore, analysis of histological alterations in different tissues of fish has been widely used for decades in aquatic toxicology to examine acute and chronic effects of various pollutants (Wester and Canton, 1991). Environmental pollution affects the quality of water and subsequently affects all living organisms in the ecosystem. Therefore, apart from identifying and managing the source of pollution, it is necessary to monitor the adverse effects of the pollutants, which reflects the health status of aquatic ecosystem (Bernet *et al*., 1999). In addition, numerous environmental risk assessment programs have approved the examination of histological alterations in different organs of fishes as biomarkers to assess the quality of aquatic ecosystems (Schmitt and Dethloff, 2000).

Histopathological modifications are the outcome of adverse biochemical and physiological changes in an organism, which implies the rapport of histopathology with physiological and biochemical biomarkers (Hinton *et al*., 1992). In all organisms, the cellular damages are contributed due to different mechanisms and factors that influence various cell types. Most of the environmental contaminants including pesticides are known to alter the activities of different enzymes, which usually results in the formation of toxic intermediates that surpasses the cellular detoxification mechanisms. Reactive oxygen metabolites produced during the detoxification process of pesticides in the liver tissue may interact with vital macromolecules such as lipid, protein, carbohydrate and nucleic acids that finally result in oxidative damage to aquatic organisms (Slaninova *et al*., 2009). Reactive oxygen species associated damages to cellular components are usually considered as important mechanism involved in the histopathological alterations (Sepici-Dinçel *et al*., 2009). Therefore, contaminant induced cytotoxicity involving cellular damages and cell death are observed morphologically as tissue necrosis, apoptosis or degenerative changes (Lesser, 2006). Environmental contaminants such as

organophosphate pesticides induce methylation and phosphorylation of cellular proteins that may reduce the reconstruction of necrotic tissues ((Murray *et al*., 2003). At the same time, contaminants with genotoxic potential that usually form DNA adducts, if not repaired properly can persists and undergo pathological changes as cell death or abnormal growth as tumor formation, which can also be detected by histopathological examinations (Balbo *et al*., 2014).

Generally, the histopathological studies have been performed to help for the establishment of underlying relationship between contaminant exposure and various biological responses in organisms. In toxicology, histological analysis have been proved as a responsive tool to detect the direct cytotoxic effects of chemical compound to target organs such as gill, liver, brain and gonads of fish in laboratory conditions (Schwaiger *et al*., 1997; Dutta, 1996; Sakr and Al-Lail**,** 2005). Moreover, it is considered as one of the cost-effective tools to detect the nature and health status of fish populations, thereby reflecting the well-being of the entire aquatic ecosystem.

1.2 Histoarchitecture of gill

Fish gills are multifunctional organ concerned with respiratory gas exchange, ion transport, acid-base regulation and excretion of nitrogenous waste materials from the body (Evans *et al*., 2005). Generally, gills account for over 50% of the surface area of fish that make it a major target tissue for most of the waterborne contaminants and thus exposure to any contaminants may directly affect gills (Playle, 1998). Histomorphological analysis demonstrates that gill is composed of primary lamellae, secondary lamellae, epithelial cells, mucous and chloride cells (Amin *et al*., 1992). Gill of the teleost fish consist of four gill arches, one on each side of the buccal cavity, which are supported by bony skeleton. The gill arch provides support to hold number of comb-like structures called gill filaments. The gill filaments extend out horizontally from the gill arches, and each gill filament produces many branches called primary lamellae (Laurent and Perry, 1991). Each primary lamella has a series of secondary lamellae positioned perpendicular to the primary lamellae. The secondary lamellae have two sheets of epithelium which are

separated by space through which blood circulates. The epithelial sheets are separated by a series of pillar cells, having a central body and are provided with extensions at each end (Wilson and Laurent, 2002). In addition to the epithelial cells, both primary and secondary lamellae are lined with specialized cell types called mucous cells, chloride cells, pillar cells, pavement cells and non-differentiated cells (Wilson and Laurent, 2002). Mucous or goblet cells are unicellular glandular cells in which the nucleus lies at the bottom. It resembles to typical goblet cells having oval or pear shaped structure with a narrow neck through which it opens outside the epithelium. It is present throughout the epithelium, including gill arch, gill filament and secondary lamellae. The secretions of mucous cells protect the fish from pathogens and contaminants and also have roles in the exchange of ions, gases and water (Laurent, 1984).

Chloride cells are large epithelial cells with light cytoplasm having numerous mitochondria. It is found along the whole lamellar epithelium of gills, however, the large quantity is found at the base and proximal parts of lamellae. Chloride cells are important for transport of chloride and sodium ions and thus mainly concerned with osmoregulatory functions (Perry, 1997). Pillar cell is a modified endothelial cell unique to the fish gill, which characterize the blood spaces within the lamellae and give the appearance of string of beads to lamellae when viewed in cross section. More than 90% of the surface area of gill epithelium is covered by squamous to cuboid-shaped cell commonly referred as the pavement or respiratory cell. The apical surface of pavement cell is usually large and polygonal called as microplicae or finger-like projections called microvilli (Laurent, 1984). Pavement cells have the typical intracellular organelles, rough and smooth endoplasmic reticulum, Golgi apparatus, lysosomes, and vesicles (Bartels, 1985). The basal and intermediate layers of the epithelium contain cells that are undifferentiated and serve as the progenitors for the other terminally differentiated epithelial cell types. However, relative to pavement cells, non-differentiated cells have more abundant free ribosomes. Desmosomes are found between non-differentiated and pavement cells whereas hemidesmosomes connect the basal undifferentiated cells to the basal lamina anchoring the epithelium (Laurent, 1984).

1.3 Histoarchitecture of liver

Liver is the largest glandular tissue of the body in which nutrients absorbed in the digestive tract are processed, stored and further utilized by other parts of the body. Liver is the major site of metabolism and also involved in various functions such as protein synthesis, storage of metabolites, bile secretion, detoxification and inactivation of xenobiotics thereby play an important role in maintaining life (Rappaport, 1963). The structural and functional unit of the liver is the acinus formed of both the hepatic lobule and portal triad. The hepatic lobule consists of the functional centre named as hepatocytes or liver cells, in which the hepatocyte-sinusoidal structures are formed. The sinusoids are capillary networks that functions as communicating channels occupied by blood cells with Kupffer cells, and are localized in the space between hepatic plates in which the hepatocytes are arranged. Based on the morphology, hepatocyte-sinusoidal structures of fish livers were classified into three different types as cord-like form, tubular form, and solid form (Figueiredo-Fernandes *et al*., 2007).

The histology of normal liver shows that the parenchyma cells are arranged to form a lattice network as polygonal cells with a central spherical nucleus and a compactly stained nucleolus. The cytoplasm of the hepatocytes was occasionally filled with fat droplets, which are neutral lipids. When fish is at cyclical starvation phases, the hepatocytes shrunk and are filled with varying amounts of yellow pigments. Based on the presence of bile duct with blood vessels, the biliary tract structures of liver were classified into four types as isolated type, biliary-arteriolar tract type, biliary-venous tract type, and portal tract type. The fish liver also contain drug metabolizing enzymes and is one of the most frequently damaged organs, but only 10% of hepatic parenchyma is required to maintain normal liver function. Studies on fish liver histopathology is increasingly been incorporated in aquatic pollution research globally, as it represent a reliable biomarker of toxic damage and carcinogen exposure (Valon *et al*., 2013). Hepatocellular foci of cellular alterations have been recommended as an early stage in the stepwise formation of hepatic neoplasia that provides an excellent example of a histopathological biomarker for toxicant exposure (Hinton *et al*., 1992).

1.4 Histoarchitecture of brain

The fundamental components in brain of teleost fish are similar to the brain of higher animals, but with differences in the form and complexity. Fish brain is generally divided into five regions namely, the anterior telencephalon, diencephalon, mesencephalon, metencephalon or cerebellum, and myelencephalon or medulla oblongata (Speare and Frasca, 2006). The telencephalon is the anterior most portions concerned with olfaction, memory, feeding behaviour, color vision and reproductive behaviour (Roberts and Ellis, 2001). Telencephalon consists of two parts, the anterior paired olfactory bulb and posterior two large cerebral hemispheres, which are collectively known as forebrain. The olfactory lobes are slender anteriorly projected bulb known as olfactory bulbs with mass of nerve cells that transmit smell impulses to other centres (Wullimann *et al*., 1996). The second part of the telencephalon is the solid mass-like paired structures which are jointed on mid-line called cerebral hemisphere. There are large bundle of nerve fibres that connects the two hemispheres anteriorly and is known as anterior commissure. The cerebral hemispheres are enclosed by a non-nervous membranous sheath called pallium, which is located dorsolaterally (Speare and Frasca, 2006).

The diencephalon is covered dorsally by the posterior bulging of the cerebral hemispheres. The cavity of diencephalon is called third ventricle, having dorsal epithalamus, lateral thalamus and ventral hypothalamus. The epithalamus contains a choroid plexus in its roof. It has two ganglionic masses, the nerve fibres from the telencephalon and also certain groups of nerve cells called habenula, which connects with the thalamus, hypothalamus and the olfactory areas of the telencephalon. The posterior part of the roof elevates to form pineal gland or epiphysis. The lateral wall of the diencephalon is called thalamus that acts as a relay centre for transmitting olfactory impulses to thalamomedullar and thalamospinal tract (Lisney and Collin, 2006). The hypothalamus is the largest and most important part of diencephalon, which constitutes the floor of the diencephalon. The ventral part of diencephalon projects a pouch-like down growth known as the infundibulum possessing pituitary gland at the tip. Overall, the diencephalon acts as an important correlation centre for

maintaining internal homeostasis, and the hypothalamus also plays an important role in controlling the endocrine system through pituitary gland (El-Bab, 2006).

The mesencephalon or midbrain is comparatively large, made up of the dorsal optic tectum and the ventral tegumentum. The ventricle communicates with mesencephalon by a narrow cavity aquaductus mesencephali or aqueduct of Sylvius. The hindbrain composed of metencephalon and myelencephalon. The metencephalon or cerebellum consists of cortex and medulla in which the surface is formed by the cortex having three layers, namely molecular layer, the Pukinje's cell layer and the granular cell layer. The cavity of the cerebellum is called metacoel and is mainly involved in the control of swimming equilibrium, maintenance and co-ordination of muscular tonus, and orientation in space (El-Bab, 2006). The myelencephalon or medulla oblongata is the most important part of the brain, which continues to the spinal cord. The cavity of medulla oblongata is called fourth ventricle and are enlarged with the development of various senses. Similar to mammals, the myelencephalon in fishes is also concerned with autonomic or involuntary functions (El-Bab, 2006).

1.5 Histoarchitecture of testes

The testis is covered by a very thin and delicate membrane called peritoneum and the wall of testis is called tunica albuginea, which lies immediately beneath the peritoneum. It is made up of thick fibrous connective tissue which protects the testis from external disturbances (Guraya, 1994). Histological examination of testes contains different stages of sperm cells within small cavity like structure called lobules. The inner part of testes is divided into several lobules of unequal size separated by means of fibrous connective tissue called septa that run inwardly from the tunica. The interstitial spaces are occupied by Leydig cells, which are involved in the production of male hormones (Grier, 1981). The lobular compartment contains Sertoli cells and germ cells, where Sertoli cells are essential for regulating the blood-testis barrier (Nagahama, 1983) and supports germ cell functions (Schulz and Miura, 2002). Primary germ cells are the immature spermatogenic cells from which spermatogonia are formed by mitosis. Primary germ cells are large, round

shaped cells with prominent nucleus at the centre (Guraya, 1994). Spermatogonia are smaller than primary germ cells with more compact nucleolus. It is attached to the wall of lobule in groups and divides mitotically to form primary spermatocytes. The primary spermatocytes are smaller than spermatogonia, having little cytoplasm and dense nucleus (Guraya, 1994). Primary spermatocyte grows and upon maturity, it undergoes meiotic division to form secondary spermatocytes. There is no cytoplasm around secondary spermatocytes and the nucleus contains dense chromatin material. Secondary spermatocytes are found in large clusters inside the lumen of lobules, where they divide mitotically to form spermatids. The spermatids also have no cytoplasm but the nucleus is small, which contains concentrated chromatin. Usually spermatids occur in cluster and finally differentiate to form spermatozoa. Spermatozoa have pear shaped head with long tail often found in the lumen of lobule as parachute shaped clumps (Pathiratne and Costa, 1984).

1.6 Histoarchitecture of ovary

Histological examination of ovarian tissue commonly reveals ovum with different stages of development, which are embedded in the loose connective tissue called stroma (Wang and Ge, 2004). Ovary contains several ovarian follicles derived from the germinal epithelium, which is an aggregate of egg and epithelial cells. Oogonia present in the ovarian follicle therefore develops into oocytes and ultimately to ova. The surrounding follicular epithelial cells also grow with developing ova and gradually separated, which is responsible for nourishing the ovum (Mumford *et al*., 2007). Ovarian histology reveals the presence of different developmental stages of oocytes such as previtellogenic, early vitellogenic and late vitellogenic stages. The previtellogenic or primary growth stage is characterized by the presence of a centrally located nucleus containing a single, basophilic nucleolus. During previtellogenic growth, cortical alveoli are deposited in the peripheral oocyte cytoplasm. In early vitellogenic oocytes, the cortical alveoli are still prominent but yolk granules or yolk globules become obvious. During the process of vitellogenesis, the hepatically derived yolk precursor, vitellogenin is sequestered and packaged into the ovary through blood stream (Patino and Sullivan, 2002). At the

end of early vitellogenic stage or on the onset of late vitellogenic stage, the cortical alveoli are pushed to the periphery of the oocyte and yolk granule fill the entire volume of cytoplasm (Guraya, 1986; Selman and Wallace, 1989). The late vitellogenic oocytes are the biggest with bulky amounts of yolk vesicles and yolk granules in the cytoplasm and are enveloped by well developed granulosa and thecal cell layers (Abraham *et al*., 1984).

It is well known that fish are widely distributed species both geographically and ecologically, which range from deep sea to small mountain streams, and from mud surfaces on land to inside holes under the seabed. Similarly, the structural characteristics of vital organs such as gill, liver, brain, muscle, intestine, testis or ovary are also unique when compared to other aquatic organisms. Thus any alteration in the histomorphology of fish reflects the health status of the organisms, which in turn show an impact on the entire aquatic ecosystem. Therefore, study on histology of tissue is a proven document to understand the effects of toxicants in the ecosystem. The present study was designed to focus the sublethal effects of chlordecone for 96 h duration could alter the histomorphology of gill, liver, brain, testes and ovary of the fish, *Pseudetroplus maculatus*.

Review of Literature

The biotic integrity of an ecosystem is reflected by the quality and quantity of the living fauna (Boersema and Reijnders, 2008). The sensitivity of organisms living in different ecosystems may vary because of the ability to respond at varying environmental conditions, including pollutants. Fish may either adapt to environmental changes or exposure to pollutant, which may result in mortality or development of different pathological conditions even at sublethal concentrations. Fish are highly susceptible to environmental pollutants as it is in immediate contact with contamination and can undergo frequent changes in the physiology particularly on biochemical, molecular, and histological parameters (Sheehan, 1984). It has also been associated with outbreaks of several diseases, low productivity and mortality in both natural as well as in laboratory conditions (Johnson and Katavic, 1984). Thus to manage healthy fish populations and ecosystems, it is required to identify the signs of damages in exposed organisms. Several biomarkers are available to detect cellular, physiological, biochemical or molecular alterations caused by environmental contaminants in animals. Among this, histopathological analysis provides one of the direct and reliable evidence by identifying the cellular destructions (Wester and Canton, 1991). Apart from other biochemical or molecular endpoints, histopathology facilitates direct cellular examination of specific target organs including gills, liver, kidney, brain and gonads which are responsible for vital functions, such as respiration, accumulation and biotransformation of xenobiotics, excretion, neural coordination and reproduction in fish within a short period of time at cheap cost (Gernhofer *et al*., 2001). Moreover, variations found in the organs are usually easier to identify by comparison with the functional ones and serve as warning signs of injury (Hinton and Lauren, 1990; Fanta *et al*., 2003).

Recently, ecotoxicological studies make use of histopathological examination as biomarkers in the evaluation of the health of fish exposed to contaminants, both in laboratory and field studies. *Labeo rohita,* when exposed to sublethal concentrations of hexachlorocyclohexane for 45 days have been shown to induce cellular alteration in different tissues such as gill, liver, kidney, skin, muscle, heart and brain (Das and Mukherjee, 2000). Cytoplasmic vacuolization, atrophy and necrosis were reported in the liver of *Anguilla anguilla,* when exposed to different

concentrations of benzopyrene, dehydroabietic acid and bleached kraft pulp mill effluent (Pacheco and Santos, 2002). The glyphosate herbicide, roundup at different sublethal concentrations for 96 h has been found to cause lamellar cell hyperplasia, lamellar fusion, epithelial lifting and aneurysm in gills, vacuolation and nuclear pyknosis in hepatocytes and lesions in kidney of Nile tilapia (Jiraungkoorskul *et al*., 2002). The pesticides, carbofuran and cypermethrin at different concentrations are known to induce histological alterations such as necrosis, hyperplasia, disintegration and individualization of hepatocytes in *Labeo rohita* (Sarkar *et al*., 2005). Similarly, malathion exposure at 0.4mg/L has been observed to cause hyperplasia, thickening of basal membrane, clubbing and fusion of secondary lamellae in the gill of gilthead seabream within 96 h of exposure (Rosety *et al*., 2005). *Cirrhinus mrigala* when exposed to sublethal concentrations of dichlorvos for 10 days showed hyperplasia, necrosis, epithelial lifting, edema, lamellar fusion, collapsed curly secondary lamellae with aneurism in gill as well as swelling of hepatocytes, congestion, vacuolar degeneration, karyolysis, dilation of sinusoids and nuclear hypertrophy in the liver (Velmurugan *et al*., 2009).

In field studies, histopathological examination is one of the frequently used biomarkers for the assessment of ecological quality of aquatic ecosystems. Accidental discharge of lindane into the Barbate River caused histopathological alterations in gill, liver and kidney of *Mugil*, *Cyprinus* and *Barbus* (Ortiz *et al*., 2003). Histopathological alterations such as inflammatory lesions, hepatocellular adenoma, intersex in gonads and cellular degenerative changes in gill and kidney has been reported in *Platichthys flesus*, *Pomatoschistus minutus* and *Zoarces viviparus*, captured from four British estuaries that implies the biological effects of contaminants (Stentiford *et al*., 2003). The neotropical fish *Prochilodus lineatus*, subjected to *in situ* tests for 7 days in a disturbed urban stream of Brazil have showed histological changes in gill, liver and kidney, when compared to the reference fish (Camargo and Martinez, 2007).

Histopathological changes in the gill of fishes due to pesticides and other contaminants have been reported by several authors globally. Deltamethrin exposure at 0.029 mg/L and 0.041 mg/L have been found to alter the histology of gills of

Cyprinus carpio, which is evidenced by lifting of the lamellar epithelium, oedema, epithelial hyperplasia, fusion of secondary lamellae, necrosis and aneurism in secondary lamellae (Cengiz, 2006). Deltamethrin exposure also induced morphological alterations such as hyperemia, fusion of secondary lamellae and telangiectasis in the gill of *Oreochromis niloticus* at 5 μg/L concentration (Yildirim *et al*., 2006). Cellular alterations like epithelial hyperplasia and necrosis, aneurism, epithelial lifting, oedema, lamellar fusion and curling of secondary lamellae has been reported in the gill of *Cirrhinus mrigala* when exposed to monocrotophos (Velmurugan *et al*., 2007). Similar cellular alterations have been reported in *Channa punctatus* when exposed to chromium (Mishra and Mohanty, 2008).

Monitoring histological changes in fish liver is a highly sensitive and accurate way to assess the effects of xenobiotic compounds in field and experimental studies. Anomalies such as cytoplasmic vacuolation, irregular shaped hepatocytes and laterally placed nucleus were observed in *Corydoras paleatus* contaminated by organophosphate pesticides (Fanta *et al*., 2003). Deltamethrin caused hypertrophy of hepatocytes, significant increase of kupffer cells, circulatory disturbances, focal necrosis, fatty degeneration, nuclear pycnosis and narrowing of sinusoids in the liver of *Gambusia affinis* (Cengiz and Unlu, 2006). Degenerative changes in the hepatic cytoplasm and nuclei, as well as hyperemia in liver were reported in rainbow trout, *Oncorhynchus mykiss* and Atlantic salmon, *Salmo salar* when exposed to 7.5 and 10 mg/L roundup exposure for 6, 26 and 96 h durations (Langiano-Vdo and Martinez, 2008). Similarly, infiltration of leukocytes, fatty degeneration, severe fat vacuolation, necrosis and darkly stained specks of necrotic nuclei has been noticed in the liver of catfish exposed to glyphosate at sublethal concentrations for 96 h (Ayoola, 2008). Similarly, di(2-ethylhexyl)phthalate at different concentrations for 96 h has been known to induce cellular damages in the gill and liver tissues of *Oreochromis mossambicus* (Revathy and Chitra, 2015). Sublethal concentrations of lead and cadmium have been shown to induce hepatic degeneration and severe necrosis in *Cyprinus carpio communis* after 28 days of exposure (Patnaik *et al*., 2011). Presence of necrotic areas is one of the most serious alterations in liver structure that can occur under the influence of contaminants.

Necrosis is a severe histopathological abnormality in the liver of *Clarias gariepinus*, when exposed to sublethal concentrations of lindane. Along with necrosis other cellular alterations such as heterophilic infiltration, pyknosis and degenerative changes were also noticed in *Clarias* (Adesina *et al*., 2006). Incidence of necrosis has been considered as an outcome of enzymatic inhibition, damages in the cellular membrane integrity and disturbances in the synthesis of proteins and carbohydrate metabolism (Manahan, 1991). Presence of necrosis in the liver of fish is also related with increase in the concentration and accumulation of xenobiotics during the detoxifying process (Ayoola, 2008).

Hepatic histopathological examination also provides important information for the development of neoplasia in fish by studying different types of foci of cellular alterations. The presence of pre-neoplastic and neoplastic toxicopathic lesions, hepatic foci of cellular alteration and hepatocellular adenoma has been observed in *Platichthys flesus, Pomatoschistus minutus* and *Zoarces viviparus* captured from British estuaries (Stentiford *et al*., 2003). Long-term exposure to creosote-contaminated sediments resulted in development of altered hepatocellular foci and hepatic neoplasms in mummichogs, *Fundulus heteroclitus* (Vogelbein and Unger, 2006). Adult killifish collected from Elizabeth River contaminated with polycyclic aromatic hydrocarbons showed a greater prevalence of hepatic and pancreatic tumors, which is evidenced by the occurrence of numerous hepatic foci of cellular alteration (Wills *et al*., 2010). The liver of *Tilapia zilli* and *Solea vulgaris* collected from Lake Qarun showed focal area of necrosis and fibrosis, vacuolar degeneration in the hepatocytes, dilation and congestion in blood sinusoid and aggregations of inflammatory cells between the hepatocytes (Mohamed, 2009).

Histopathological examination of nervous system is an important component of neurotoxicology (Jortner, 2005). Degeneration of neuronal cells, swelling of pyramidal cells, vacuolization and dystrophic changes has been observed in the brain of *Cyprinus carpio* when exposed to lead and cadmium for 28 days (Patnaik *et al*., 2011). *Labeo rohita* fingerlings, when exposed to zinc metal at 5 and 10 ppm for 5 and 15 days has been shown to cause enlarged pyramidal cells with extensive vacuolation in the brain tissue (Loganathan *et al*., 2006). Malathion exposure to

Ophiocephalus punctatus has been shown to cause severe damages to brain tissue as disintegrated neural cells at different concentrations (Pugazhvendan *et al*., 2009). Fenitrothion at sublethal concentrations caused hyperemia in the brain tissue along with histopathological alterations in gill, liver, kidney and testes of *Oreochromis niloticus* (Benli and Ozkul, 2010). Severe necrosis has been observed in the brain of *Channa punctatus* exposed to endosulfan for 96 h along with inhibition of acetylcholinesterase activity (Sarma *et al*., 2010). In another study, acute, subchronic exposure of chlorpyrifos caused degeneration, detachment, necrosis and vacuolization in different regions of the brain of *Channa punctatus* (Mishra and Devi, 2014).

Gonadal histology is a routinely used tool for identifying different stages of development, sex verification, documenting presence of intersex etc. Along with hormone and vitellogenin measurements, gonadal histology is employed in morphological and fecundity studies that can provide insights into the effects of various environmental stressors on reproductive health (Blazer, 2002). Gonadal histopathology provides evidences for sexual disruption in gudgeon, *Gobio gobio* collected from the Rivers Aire and Lea of England. Histological examination of the gonads revealed that few of the male fish population were intersex, as defined by the simultaneous presence of both testicular tissue and ovarian tissue, specifically oocytes (Van Aerle *et al*., 2001). Administration of ethinylestradiol and methyltestosterone to juvenile zebrafish resulted in skewed sex ratio involving occurrence of intersex and altered gonadal developmental stages, which has been demonstrated by histopathological examinations (Orn *et al*., 2003). In another study, Russian sturgeon fish with intersex gonads has been demonstrated by employing histopathological assessment. Gonadal histology of the fish revealed that intersex gonads were typical female ovaries with one or more testicular components embedded. Moreover, the histological examination in sturgeon fish also showed that the ovarian component of the intersex gonad was at the pre-vitellogenic stage as in normal females, and the testis component contained spermatids and mature spermatozoa as in normal males (Jackson *et al*., 2006).

The ovary of *Ophiocephalus punctatus* showed inhibited oocyte development with degenerative changes, broken germinal vesicles and unevenly distributed yolk after exposure to malathion at different sublethal concentrations for 7 days (Pugazhvendan *et al*., 2009). The lipophilic UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate exposure has been shown to decrease the number of spermatocytes in the testes and primary oocytes in the ovary with more vitellogenic oocytes in fathead minnows (Christen *et al*., 2011). Wild fishes sampled from downstream regions of municipal wastewater discharges showed the testes with higher frequency of earlier stages such as spermatogonia and spermatocytes and lower percentage of spermatozoa when compared to the fishes from reference site, which demonstrate the effect of contaminants on the reproductive potential of the fish (Tetreault *et al*., 2011). Red tilapia when exposed to cadmium showed separation of follicular membrane from underlying ooplasm, abnormal vacuolization of ova, inhibited spawning and impaired ovarian development in females as well as degeneration of spermatogenic cells, fibrosis of lobule walls, blood infiltration and significant decrease in sperm number and sperm motility in male fish (El-Ebiary *et al*., 2013).

Acute exposure to malathion caused reduction in size of mature oocytes, disruption and vacuolation in cytoplasm in *Channa punctatus*. However, chronic exposure leads to complete loss of normal configuration of ovary, necrosis, elongated ovarian follicles and fragmented ova with abnormal shape (Magar and Bias, 2013). The testicular and ovarian histology has been completely damaged in *Labeo rohita* when exposed to sublethal concentration of endosulfan for long-term duration. The testes is characterized by damaged connective tissue, fewer Leydig cells, damaged Sertoli cells and less pronounced seminiferous tubules whereas ovary showed clumping of cytoplasm, degeneration of follicular cells, atretic oocytes, fusion of oocytes together with loose and ruptured follicular epithelium (Archana and Shashikant, 2014). Similarly, diazinon exposure in common carp showed degeneration, congestion and fibrosis in testis and adhesions between oocytes and necrosis in the ovary (Korkmaz and Donmez, 2017).

Materials and Methods

3.1 Experimental design

Adult male and female freshwater cichlid fish, *Pseudetroplus maculatus* were exposed to chlordecone at two sublethal concentrations i.e., 3.5 and 7 μg/ L for 96 h. Ten animals were maintained in both treatment concentrations, and also in positive (1% DMSO-vehicle) and negative control groups.

3.2 Post-treatment analysis

At the end of every treatment period, fish were killed and non-reproductive (gill, liver and brain) and reproductive (testis and ovary) tissues were removed. Tissues were fixed in 10% buffered formalin for 24 h to 48 h for histological examinations. Tissues were then dehydrated in ascending grades of ethanol and were cleared in xylene until they became translucent. Tissues were transferred to molten paraffin wax for 1 h to remove xylene completely and impregnated with wax. The blocks were cut and sections of thickness 4 to 6 microns were prepared using rotary microtome. The sections were stained with haematoxylin and eosin and mounted in DPX (Roberts and Smail, 2001). The slides were examined for structural alterations under light microscope and compared with that of control tissues. Photomicrographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope-2 Plus Trinocular Research Microscope.

Results

4.1 Effect of chlordecone on histopathology of gill

Histological examination revealed that the positive control tissues of gill, i.e., treated with 1% DMSO (vehicle) are found similar to the negative control gill tissue with normal histoarchitecture. Chlordecone when exposed at both sublethal concentrations (3.5 and 7μg/L) for 96 h showed upliftment of gill epithelium, hyperplasia, edema and absence of secondary lamellae in gill of fish (Figure 1).

4.2 Effect of chlordecone on histopathology of liver

DMSO (vehicle control)-treated fishes showed no changes in the histomorphology of hepatocytes and it was similar to the negative control tissues. Hepatocytes of chlordecone-treated fish at both sublethal concentrations showed severe damages such as cytoplasmic vacuolization and complete necrosis (Figure 2).

4.3 Effect of chlordecone on histopathology of brain

Histomorphological changes on brain tissue showed cerebral edema, necrosis of neurofibrillar region, vacuolization and nuclear pyknosis (Figure 3). The severity of damage increased when the concentration of chlordecone was increased. No structural alteration was noticed in DMSO-treated brain tissues (Figure 3).

4.4 Effect of chlordecone on histopathology of testis

Histological examination revealed control testis having compact seminiferous tubules with well-developed spermatogonia, spermatocytes and mature spermatozoa enclosed in the lumen of testis (Figure 4). Chlordecone exposure at one-tenth of sublethal concentration caused degeneration of spermatogenic cells and one-fifth of sublethal concentration showed gross vacuolization, distortion of seminiferous epithelium in seminiferous tubules followed by reduced number of spermatocytes and spermatozoa and complete atresia (Figure 4).

4.5 Effect of chlordecone on histopathology of ovary

Normal histology of ovary showed vitellogenic oocytes characterized by presence of yolk vesicles and yolk granules in the cytoplasm (Figure 5).

Chlordecone treatment at low sublethal concentration showed gross vacuolization in the developing oocytes. However, when the concentration is increased showed degenerated and loosely packed vitellogenic oocytes with highly proliferated connective tissues and damaged yolk granules, vacuolization and highly reduced atretic oocytes (Figure 5).

Figure 1: **Photomicrographs of histological sections, stained with hematoxylin and eosin in the gill of *Pseudetroplus maculatus*. A-Control; B-DMSO-treated; C and D-Chlordecone-treated for 96 h at 3.5 µg/L and 7 µg/L, respectively, showing upliftment of gill epithelium (*); edema in gill lamellae (arrow)**

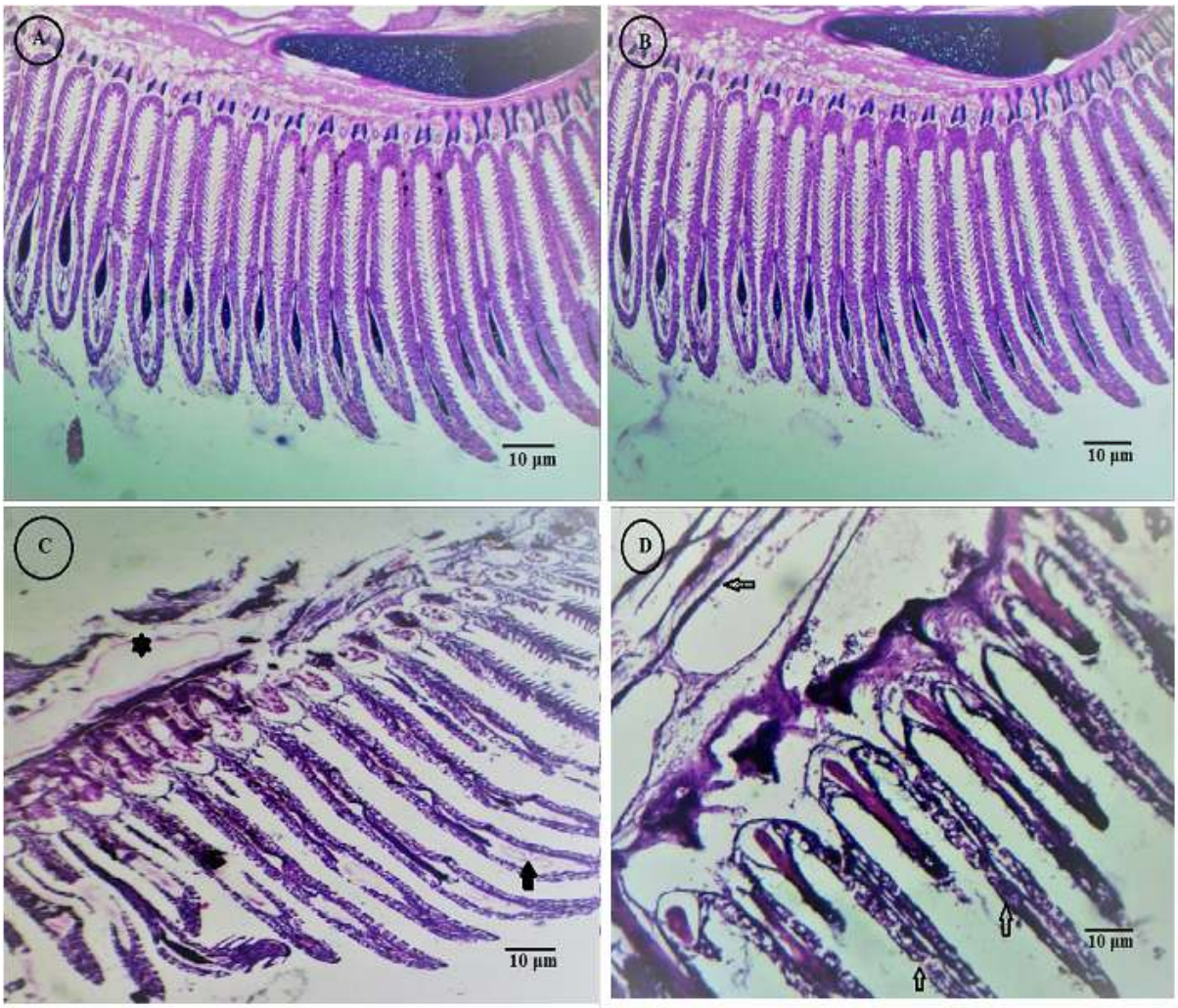


Figure 2: **Photomicrographs of histological sections, stained with hematoxylin and eosin in the hepatocytes of *Pseudetroplus maculatus*. A-Control; B-DMSO-treated; C and D-Chlordecone-treated for 96 h showing vacuolization (V) and complete necrosis (←) at 3.5 µg/L and 7 µg/L, respectively**

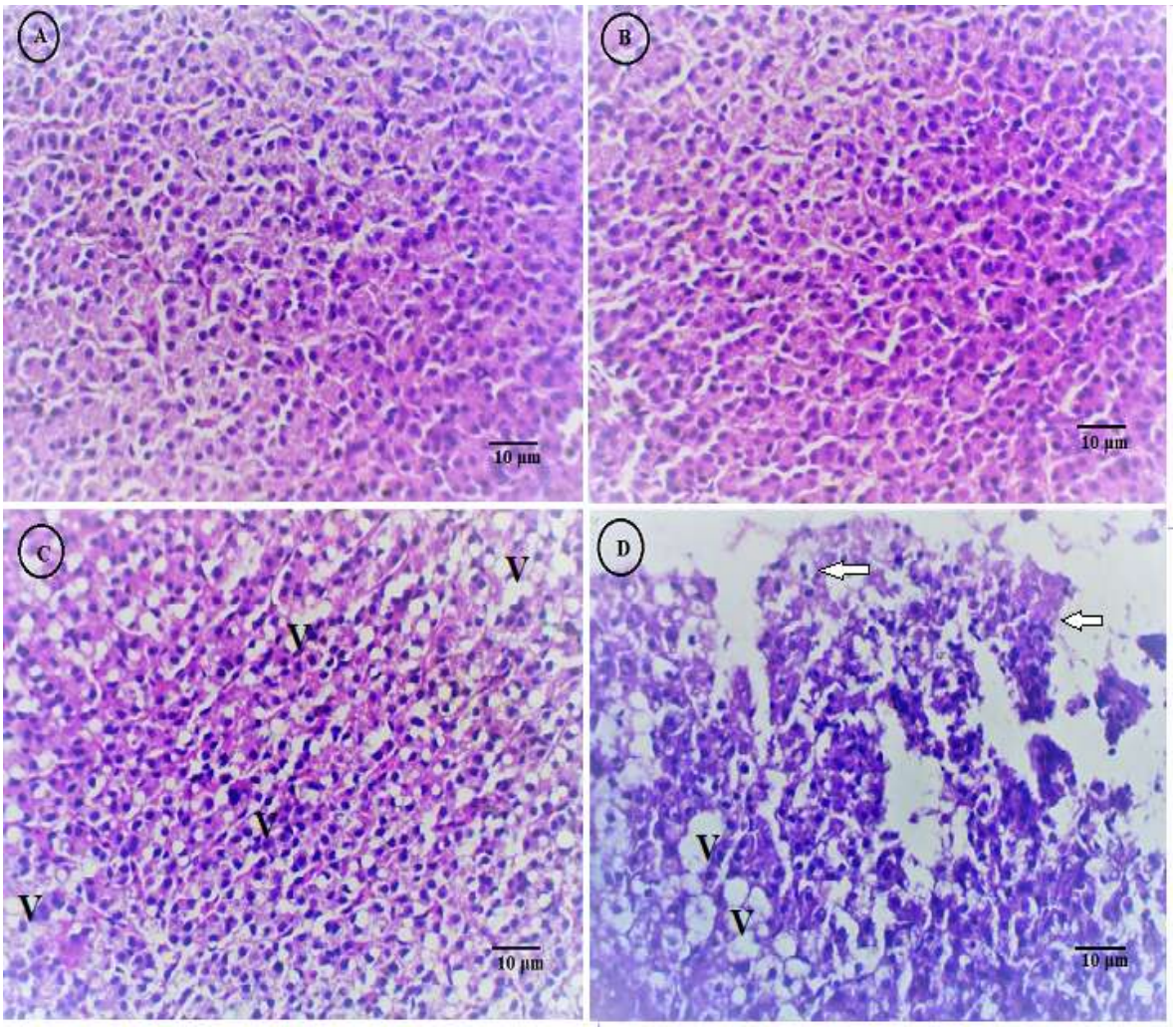


Figure 3: **Photomicrographs of histological sections, stained with hematoxylin and eosin in the brain of *Pseudetroplus maculatus*. A-Control; B-DMSO-treated; C and D-Chlordecone-treated for 96 h showing necrosis (arrow) and vacuolization (V) at 3.5 µg/L and 7 µg/L, respectively**

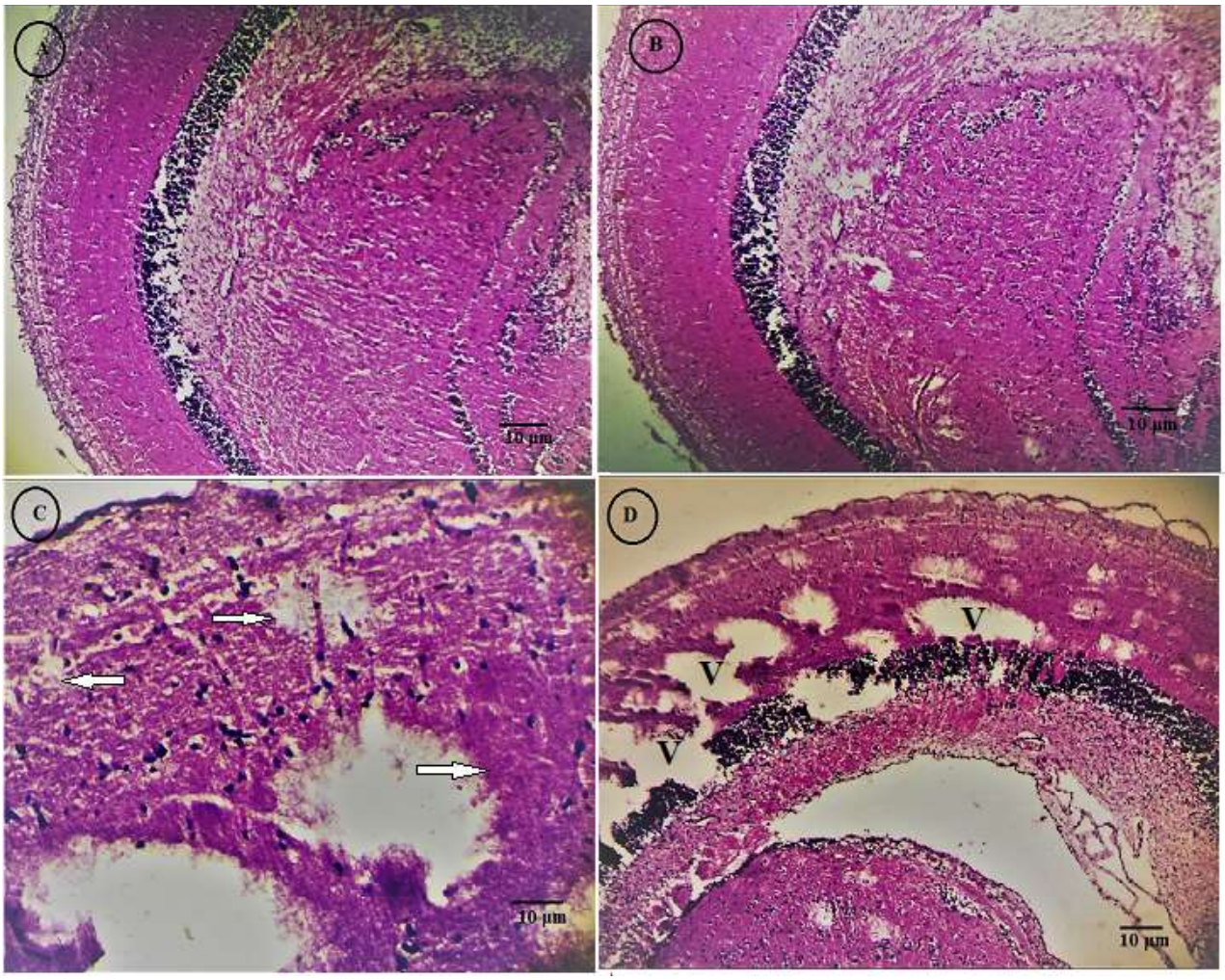


Figure 4: **Photomicrographs of histological sections, stained with hematoxylin and eosin in the testis of *Pseudetroplus maculatus*. A-Control; B-DMSO-treated; C and D-Chlordecone-treated for 96 h showing showing degeneration of spermatogenic cells (D), reduced number of spermatocytes and complete atresia (A) at 3.5 µg/L and 7 µg/L, respectively**

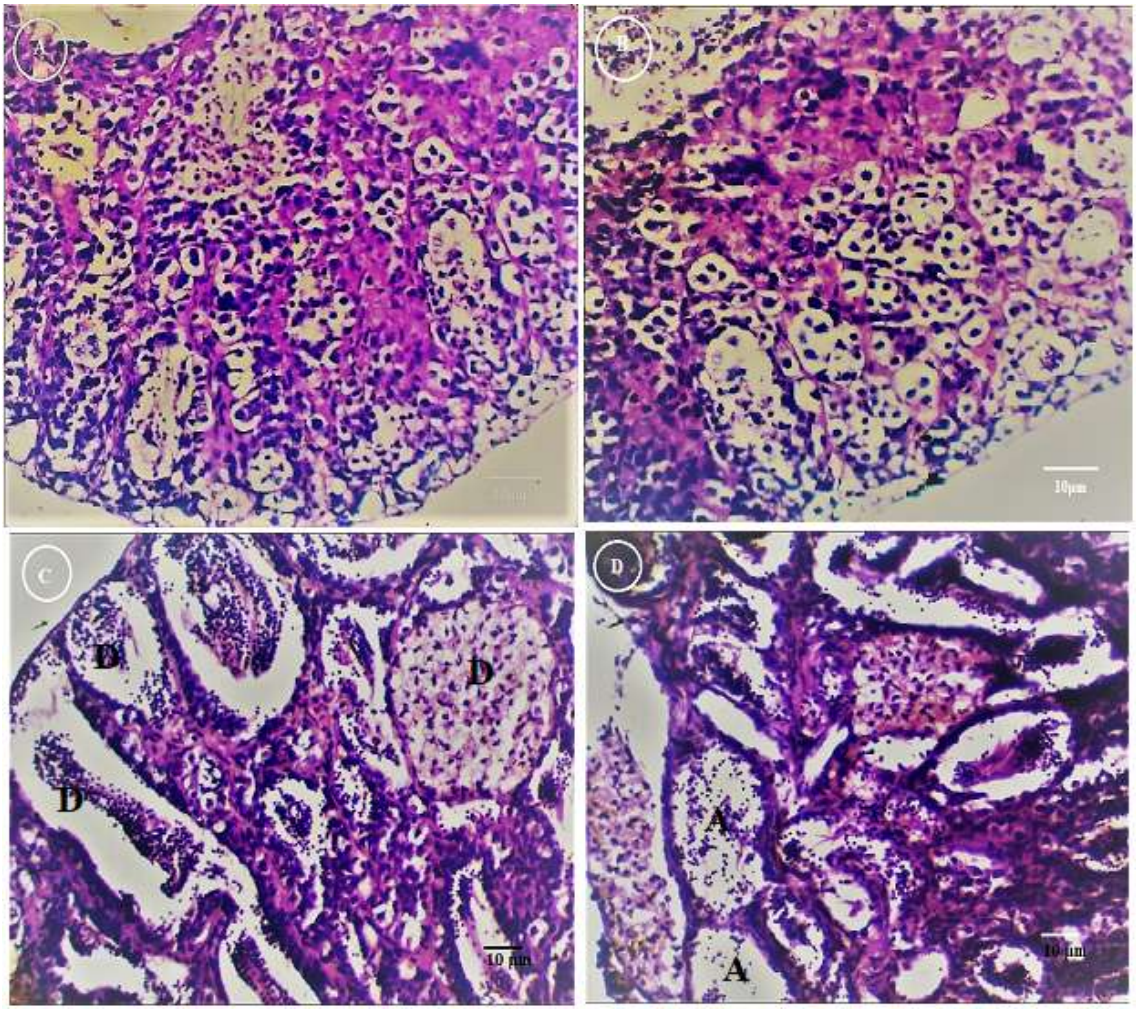
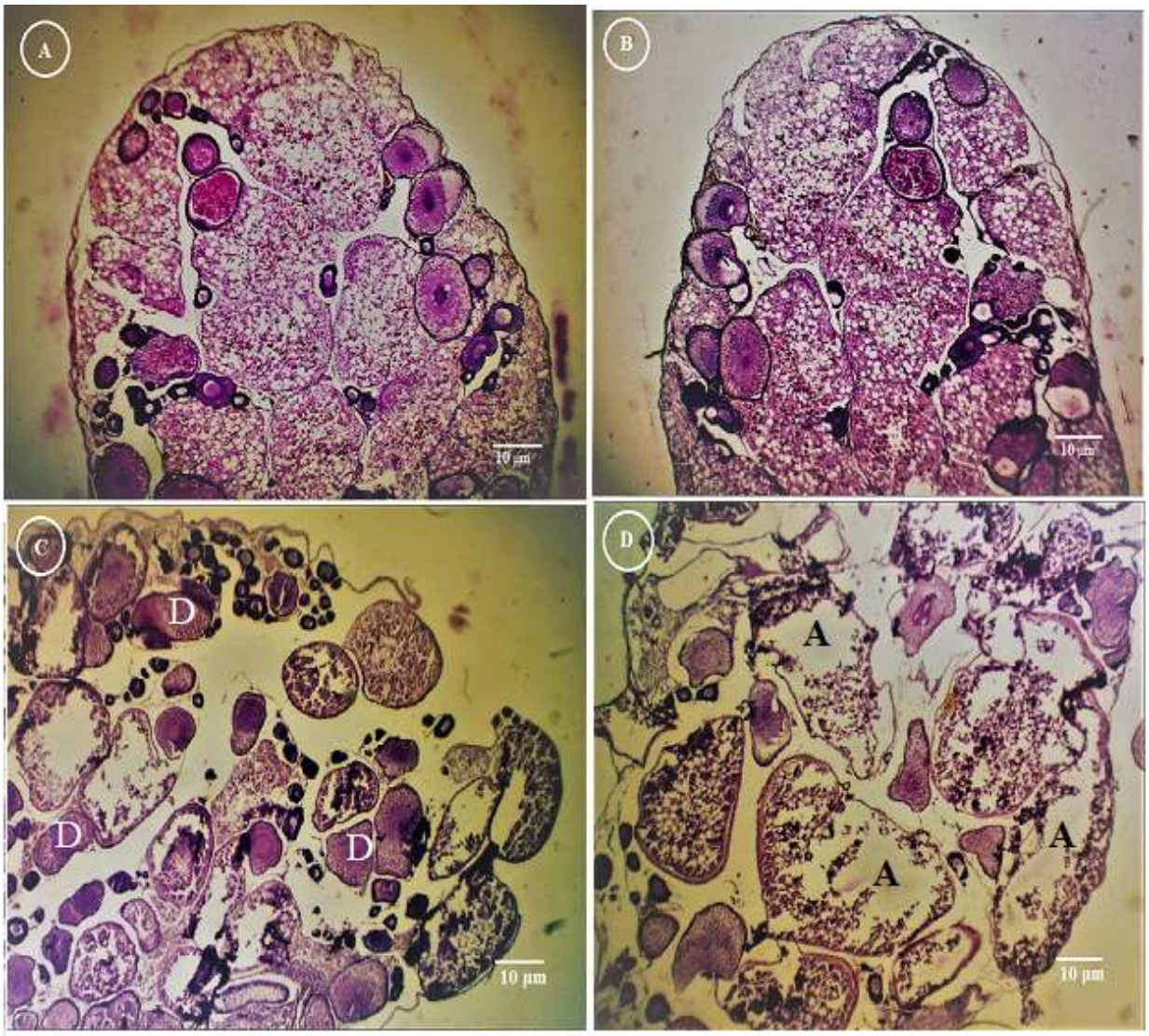


Figure 5: **Photomicrographs of histological sections, stained with hematoxylin and eosin in the ovary of *Pseudetroplus maculatus*. A-Control; B-DMSO-treated; C and D-Chlordecone-treated for 96 h showing showing vitellogenic oocytes with damaged yolk granules (D) and completely deformed and atretic oocytes (A) at 3.5 µg/L and 7 µg/L, respectively**



Discussion

5.1 Effect of chlordecone on the histopathology of gill

The occurrence of man-made and natural environmental contaminants in the aquatic environment has become a growing concern in recent years. Adverse effects of environmental toxicants induce histopathological changes in fish. Histology is a useful tool to assess the degree of pollution and it represent important biomarker for the diagnosis of pollution in aquatic ecosystem, especially the sublethal effects of toxicants. In the present study, the gill tissues of control fishes showed normal architecture having complete gill arches with compact primary and secondary lamellae. Chlordecone exposure at sublethal concentrations i.e., 3.5 and 7µg/ L for 96 h induced marked pathological alterations in the gill of *Pseudetroplus maculatus*. Chlordecone treatment at 3.5µg/ L showed upliftment and separation of gill epithelium from the gill lamellae and hyperplasia in the gill arches. Alterations like epithelial lifting, hypertrophy and hyperplasia of the epithelial cells are examples of primary defense mechanisms of the gill tissue (Mallatt, 1985). The lifting of the lamellar epithelial cells away from the basement membrane is one of the adaptations of gill tissues to reduce the distance of inter-lamellar tissues from the exposed toxicant and also decrease the rate of respiratory gas exchange by raising the diffusion (Wood, 2001; Au, 2004). Similar results of gill epithelial uplifting has been observed in carp and *Pseudetroplus maculatus* when exposed to effluents of wastewater treatment plant and fullerene C_{60} nanoparticles, respectively (Coutinho and Gokhale, 2000; Sumi and Chitra, 2017).

Hyperplasia is also one of the adaptive modifications by the tissue to protect the underlying cells from the entry of the contaminant (Meissner and Diamandopoules, 1977). However, hyperplasia of epithelial cells and mucous cells leads to the fusion of secondary lamellar cells, which in turn decrease the surface area available for oxygen exchange and increase the oxygen diffusion across water and blood (Skidmore and Tovell, 1972). Though hyperplasia has protective functions, it may also be an obstacle to the respiratory, excretory and secretory functions of gill tissue (Eller, 1975). Chlordecone exposure at 7µg/ L concentration for 96 h caused fusion of secondary lamellae, edema in the primary lamellae and absence of secondary lamellae in few areas. The disappearance of secondary

lamellae affect the free gas exchange and are also associated with disturbance of blood flow, and therefore affect the vital functions of gill tissue as respiration, osmoregulation and excretion (Fernandes and Mazon, 2003). The present results are in agreement with the toxic effects of one of the pesticides, chlorpyrifos in the fish, *Pseudetroplus maculatus* (Raibeemol and Chitra, 2016). Similarly, degenerative changes in gill such as intraepithelial edema in the secondary lamellae, erosion of secondary lamellae, thickening of lamellae, inflammation of epithelial cells, breakages in primary lamellae, necrosis and rupture of gill epithelium has been reported during the exposure of monocrotophos at sublethal concentrations in mosquito fish (Rao *et al*., 2005). Exposure to atrazine at 0.18 mg/L for 120 h resulted in epithelial hyperplasia, vacuolization, degradation of epithelial cells, secondary lamellae and pillar cells in *Labeo rohita* (Jayachandran and Pugazhendy, 2009). Sublethal exposure to lindane has been shown to induce hyperplasia of the epithelial lining of the secondary lamellae, abnormal raising and swelling of the epithelium, necrosis and shortening of the secondary lamella, fusion of the secondary lamellae and excessive mucous secretion in the gill of *Etroplus maculatus* (Nandan and Nimila, 2012). Therefore, the present results coincide with other literatures stating that chlordecone exposure at sublethal concentrations for 96 h cause modifications in gill architecture of the fish thereby affecting the vital functions.

5.2 Effect of chlordecone on the histopathology of liver

The present study showed discrete morphological alterations in heptocytes of fish treated with two sublethal concentrations of chlordecone and the degree of pathology increased in concentration-dependent manner. Cytoplasmic vacuolization and degenerative changes were observed in the hepatocytes after one-tenth of sublethal concentration of chlordecone which leads to the disruption in the lattice arrangement of hepatocytes. Formation of vacuole is a cellular defensive mechanism against substances detrimental to hepatocytes and is responsible for collecting the harmful elements and removing from interfering with the biological activities of the normal cells (Mollendroff, 1973). Vacuolization also indicates the imbalance

between the rate of synthesis and release of substances in the parenchymal cells into the systemic circulation (Gingerich, 1982). Increased vacuolization is the common response of hepatocytes owing to liver injury and are likely due to the accumulation of glycogen in hepatocytes (Wester and Canton, 1986). Vacuolization of hepatocytes is also considered as a sign of degenerative process possibly due to metabolic damage caused by contaminant exposure (Pacheco and Santos, 2002). Generally, the cytoplasmic vacuoles of hepatocytes contain lipids and glycogen, which are concerned with normal metabolic function of the liver (Camargo and Martinez, 2007). Vacuolation of hepatocytes has been reported in mrigal carp, *Cirrhinus mrigala* and catfish, *Corydoras paleatus*, when exposed to pesticides lambda-cyhalothrin and methyl parathion, respectively (Fanta *et al.*, 2003; Velmurugan *et al.*, 2007). Similarly, hydropic vacuolation has been observed in winter flounder, *Pleuronectes americanus* sampled from chemically contaminated habitats (Moore *et al.*, 1997).

Exposure of chlordecone at 7µg/L concentration is characterized by complete necrosis and disorganization of hepatocytes. Hepatic necrosis or atrophy and disorganized hepatocytes are the frequently occurring tissue damage upon contaminant exposure that could be due to the accumulation of xenobiotics in the liver tissues. Moreover, necrosis is also contributed by the excessive effort exerted by the fish to get rid off the toxicant during detoxification (Olufayo and Alade, 2012). Cytotoxicity study has shown that necrosis is caused by reactive oxygen intermediates and oxidative stress (Pan *et al.*, 2009). Thus, it is confirmed that induction of tissue damages could be due to the altered metabolic functions of the liver or induction of oxidative stress by chlordecone exposure in *P. maculatus* (refer chapter 5 and 6). Oxidative stress induced necrotic and apoptotic damages have been reported in the liver of *Cyprinus carpio* when exposed to sublethal concentration of titanium dioxide nanoparticles (Linhua *et al.*, 2009). Similarly, atrazine and chlorpyrifos exposure caused induction of oxidative stress and hepatic tissue damages such as necrosis, vacuolation, pyknotic nuclei, hypertrophy and fatty infiltration in common carp (Xing *et al.*, 2012). Moreover, it is clearly demonstrated

that necrosis is responsible for the decreased hepatosomatic index of *P. maculatus* upon chlordecone exposure, which was discussed in chapter 5.

5.3 Effect of chlordecone on the histopathology of brain

The histoarchitecture of brain in the control fish showed clear neural cells with distinct nuclei. Chlordecone exposure at both sublethal concentrations induced pathological changes in the brain of exposed fish showing cerebral edema, necrosis of neurofibrillar region, vacuolization, nuclear pyknosis and degenerative changes in concentration-dependent manner. The present results are in agreement with another study that exposure to endosulfan for 96 h induced histopathological changes in the brain of *Channa punctatus* (Sarma *et al*., 2009). Similar results were noticed in *Cyprinus carpio* when exposed to sublethal concentration of quinalphos where the severity of brain damages increased with duration of exposure (Chamarthi *et al*., 2014). *Clarias gariepinus* exposed to different concentrations of glyphosate has been shown to cause severe damages in the brain, characterized by degeneration of dark-stained purkinje neurons, edema and vacuolation (Erhunmwunse *et al*., 2014). In the present study the possible reduction in the cholinergic activity of brain could be correlated to the neuronal damages induced as a result of chlordecone exposure. Chlordecone is a potent neurotoxic agent, which is known to inhibit the activity of acetylcholinesterase enzyme in the brain of *P. maculatus* and also associated with the change in the behaviour of the fish, which was discussed in chapter 1 and 5.

5.4 Effect of chlordecone on the histopathology of testes

In the present study, histological examination revealed control testis having compact seminiferous tubules with well-developed spermatogonia, spermatocytes and mature spermatozoa enclosed in the lumen of testis. Chlordecone exposure at one-tenth of sublethal concentration caused degeneration of spermatogenic cell, reduced number of spermatocytes in the testes after 96 h. Exposure at 7µg/L concentration of chlordecone showed distortion of seminiferous epithelium followed by reduced number of spermatocytes and spermatozoa along with atresia and necrosis. Exposure to nonylphenol, bisphenol and their mixtures for long-term

durations has reported to cause testicular distortion in *Xiphophorus helleri* (Kwak *et al*., 2001). Degeneration of seminiferous epithelium and delayed spermatogenesis with intra-tubular multinucleated giant cells has been noted in Atlantic cod, *Gadus morhua* when exposed to crude oil (Khan and Kiceniuk, 1984). Thus the alteration in the histomorphology of testis in chlordecone-treated fish represents one of the reasons for the disruption of endocrine system, alterations in testicular steroidogenesis, spermatogenesis or sperm functional parameters as sperm motility, viability and concentration as discussed in chapter 2.

5.5 Effect of chlordecone on the histopathology of ovary

Histological study of control ovarian tissue showed typical structural organization of different stages of oocytes embedded in the compact stroma. Chlordecone treatment at 3.5μg/ L concentration for 96 h showed damage in the germinal epithelium, gross vacuolization in the developing oocytes, vitellogenic oocytes with damaged yolk granules, atresia and necrosis. Similar cellular alterations such as degeneration of ovarian membrane and connective tissues and vacuolization has been observed in *Heteropneustes fossilis* exposed to sublethal concentration of carbofuran for 30 days (Chatterjee *et al*., 1997).

Malathion exposure has shown an inhibition in the growth of previtellogenic oocytes at lower concentration, while the increase in concentration resulted in degenerative changes in the ovarian follicles and rupture of ovarian epithelium (Ramachandra, 2000). In the present study, chlordecone exposure at higher concentration showed completely distorted and atretic oocytes having yolk granules scattered in the ovarian cavity. It also showed necrosis and complete disorganization in the arrangement of oocytes with different stages of development. Similar result has been reported in the ovary of bluegill, when exposed to diazinon at 60μg/L (Dutta and Maxwell, 2003). Similarly, increased incidence of atretic oocytes, distorted and disorganized ovarian follicles with necrosis and vacuolization was reported in *Oreochromis mossambicus* after exposure to DDT for 40 days (Mlambo *et al*., 2009). The occurrence of ovarian histopathological alterations could be related to the variation in the production of various reproductive hormones by

contaminant exposure, which in turn could lead to decreased production of eggs in fish (Chang and Yueh, 1990).

Thus chlordecone exposure induced histological alterations in gill, liver, brain, testes and ovary of *P. maculatus* at both sublethal concentrations. Exposure to chlordecone even at sublethal concentrations could cause severe health hazards to non-target animals in aquatic ecosystem, particularly fish. Fish are the final chain of aquatic food web and an important food source to human, so the exposure of chlordecone can be transferred into human through the food chain.Therefore, chlordecone induced severe toxicity in the fish may likely to affect the survival and maintenance of population size in natural environment.

Conclusions

1. Chlordecone induced gill tissue damage indicates disturbances in vital functions of gill tissue as respiration, osmoregulation and excretion.

2. Morphological alteration in hepatocytes of chlordecone exposed fish reflects altered metabolic functions of the liver.

3. Pathological changes in the brain of treated fish prove chlordecone as neurotoxicant.

4. Modification in the histomorphology of testis in chlordecone-treated fish represents disruption of endocrine system, alterations in testicular steroidogenesis, spermatogenesis and sperm functions.

5. Ovarian histopathological leisons could be due to the variation in the reproductive hormones leading to decrease in egg production and ultimately decline the fish population in the natural environment.

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APPENDIX-LIST OF PUBLICATIONS

- **Asifa, K. P,** Balakrishnan,V and chitra, K. C (2014). Toxicity evaluation of chlordecone and its effect on oxidative imbalance in the cichlid fish, *Etroplus maculatus* (Bloch). *International Journal of Zoology and Research,* 4 (2), 1-20.
- **Asifa, K.P**. and Chitra, K.C. (2015). Evaluation of LC_{50} and behavioural responses of bisphenol A in the cichlid fish, *Etroplus maculatus*. *International Journal of Current Research,* 7, 16725- 16729.
- **Asifa, K.P.** and Chitra, K.C. (2015). Determination of median lethal concentration (LC_{50}) and behavioural changes on the Cichlid fish, *Etroplus maculatus* exposed to chlordecone. *International Journal of Science and Research,* 4, 1473-1475.
- **Asifa, K. P,** Vidya, P.V and Chitra, K. C (2016). Chlordecone-induced changes in muscular antioxidant system of cichlid fish, *Etroplus maculatus* (Bloch, 1795). *International Journal of Research,* 3(1), 21-29.
- **Asifa, K.P.** Vidya, P. V. and Chitra, K.C. (2016). Assessment of median lethal concentration (LC_{50} - 96h) and behavioural modification of nonylphenol in the Cichlid fish, *Etroplus maculatus* (Bloch, 1795). *International Journal of Advanced Life Sciences*, 9 (2), 190-195.
- **Asifa, K.P**. and Chitra, K.C. (2016). Evaluation of nonylphenol toxicity in *Etroplus maculatus* (bloch, 1795): response on gill antioxidant defence system. *Trends in Fisheries Research*, 5 (1), 1-8.
- **Asifa, K.P**. and Chitra, K.C. (2016). Alteration in hepatic antioxidant defense system induced by nonylphenol in cichlid fish, *Etroplus maculatus* (Bloch, 1795). *The Journal of Zoology Studies*, 3(4), 30-38.
- **Asifa, K.P**. and Chitra, K.C. (2016). Impact of nonylphenol on antioxidant system and acetyl cholinesterase activity in the brain of *Etroplus maculatus* (Bloch, 1795). *International Journal of Research*, 3 (13), 114-123.
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Chapters in Book:

- **Asifa, K.P**., Vidya, P. V. and Chitra, K. C. (2018). Assessment of median lethal concentration (LC_{50}-96 h) and behavioural modification of nonylphenol in the cichlid fish, *Etroplus maculatus* (Bloch, 1795). Chapter I: Toxicology of metals and metalloids and impact on human and animals, pp 7-22. (Badal Bhattacharya, Jacob de Boer and Pasquale Avino, Eds.), In: Impact of Pollutants on Ecosystems and Human Health. A Publication of Institute of Ecotoxicology and Environmental Sciences, Kolkata-700097, West Bengal, INDIA. ISBN: 978-81-928924-5-0.

- Vidya, P. V., **Asifa, K.P**. and Chitra, K. C. (2018). Hepatic histopathology of *Oreochromis mossambicus* (Peters, 1852) under silica nanoparticles toxicity. Chapter II: Environmental toxicants and pathogens–impact on human and animal health, pp 23-40. (Badal Bhattacharya, Jacob de Boer and Pasquale Avino, Eds.), In: Impact of Pollutants on Ecosystems and Human Health. A Publication of Institute of Ecotoxicology and Environmental Sciences, Kolkata-700097, West Bengal, INDIA. ISBN: 978-81-928924-5-0.