BIOCHEMICAL EFFECTS OF ETHANOL ON *OREOCHROMIS MOSSAMBICUS* (PETERS)

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By

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***** Dedicated to

God Almighty ******

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Certificate

This is to certify that the thesis entitled "**BIOCHEMICAL EFFECTS OF ETHANOL ON *OREOCHROMIS MOSSAMBICUS* (PETERS)**" is an authentic record of the research work carried out by Ms. Smitha. V. Bhanu under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of philosophy in Biochemistry of Cochin University of Science and Technology, and no part there of has been presented for the award of any other degree, diploma or associateship in any university.

Dr. BABU PHILIP

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November 2009

DECLARATION

I hereby declare that the thesis entitled "**BIOCHEMICAL EFFECTS OF ETHANOL ON *OREOCHROMIS MOSSAMBICUS* (PETERS)**" is a genuine record of research work done by me under the supervision and guidance of Prof. Dr. Babu Philip, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology. The work presented in this thesis has not been submitted for any other degree or diploma earlier.

Smitha. V. Bhanu

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Preface

Several geopolitical factors such as negative environmental consequences of fossil fuels and concerns about petroleum supplies aggravated the worries concerned with global warming. This has fueled the search for and production of renewable sources of energy worldwide for the past few years. The increasing demand of alternative energy sources has created interest in the production of ethanol and ethanol blended fuels since the 1970's. Amendments to the Clean Air Act in 1990 opened the door for increased ethanol use. Amendments to the Clean Air Act boosted growth in the ethanol industry, and now ban on Methyl Tertiary Butyl Ether could propel it even further. The use of alcohol as a fuel was first introduced in Brazil in the year 1970. But nowadays the world's total ethanol production depends solely upon United States. Even though the usage of ethanol in vehicle as a fuel is increasing worldwide, the potential risks associated with it have not been examined. Reports stated that unburned ethanol emissions results in a global-scale source of acetaldehyde larger than that of direct emissions. But the actual fate of ethanol in the environment and its effect on aquatic organisms apart from fish kill have not been studied in detail. Like oil spills, ethanol spills also bring about deleterious effects on aquatic environments. In this study, effect on aquatic environment brought about by ethanol is discussed in detail by using *Oreochromis mossambicus* (Peters) as the animal model. This thesis studies the toxicity of ethanol using haematological, biochemical and histopathological parameters.

The present study exhibited the behavioural changes brought about by the euryhaline teleost *Oreochromis mossambicus* (Peters) when subjected to lethal toxicity studies. These changes were mainly due to respiratory stress. GC/MS study exhibited the presence of ethanol in an effluent which seemed to be close to the sub lethal dosage value selected for the study. Presence of ethanol in blood which was detected by GC seems to be dose and duration dependent. The decrease in membrane bound enzymes suggests the damages in the gill architecture. Marked alterations were exhibited in the haematological parameters studied. RBC hemolysis was increased both in *in vitro* and *in vivo* conditions. Trace elements levels such as copper, zinc and selenium were studied by atomic absorption spectrophotometry.

Decrease in lysosomal fraction of β glucuronidase and acid phosphatase activities were observed both in *in vitro* studies and *in vivo* studies. Increase in serum parameters such as AST, ALT, CK, cortisol, vitamin B12 and ferritin as well as decrease in serum protein, albumin was obtained. Biochemical parameters of carbohydrate, protein and lipid metabolism exhibited marked alterations in serum, blood and tissues of *Oreochromis mossambicus* (Peters). Decrease in Cyt.c. oxidase activity indicates the reduced availability of oxygen. The increase in serum uric acid and creatinine values refer to the renal effects of ethanol. An increase in lipoperoxidation products as well as decrease in enzymatic and non enzymatic antioxidants was observed in the tissues of *Oreochromis mossambicus* (Peters). Histopathological analysis carried out in the tissues such as gills, liver, heart and kidney proved the deleterious impact brought by ethanol. All this points to the fact that the widespread usage of ethanol ultimately pollutes the aquatic environment thereby affecting the aquatic fauna mainly fishes which have a major role in the economy of the country and serves as valuable dietary sources of protein. All the above mentioned tests are useful for evaluating the environmental hazard brought by ethanol.

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Plate10.4f Photomicrograph of the kidney of *O. mossambicus* exposed for 21 days at 0.65g/l ethanol showing adhesion (A), necrosis (N) and fibrosed area (F). (H & E × 40)

Plate10.4g Photomicrograph of the kidney of *O. mossambicus* exposed for 21 days at 1.3g/l ethanol showing thickening of bowman's capsule (TBC), intercapillary wall thickening (IWT) and shrinkage of glomeruli (SG). (H & E × 40)

Plate10.4h Photomicrograph of the kidney of *O. mossambicus* exposed for 21 days at 2.6g/l ethanol showing glomerular thickening (GT), tubular necrosis (TN), glomerular necrosis (GN) and thickening of bowman's capsule (TBC). (H & E × 40)

List of Notations and Abbreviations

%	percent
×	Interaction effect
µg	microgram
µg/dl	microgram per decilitre
µl	micro litre
µM	micromolar
µmol	micromole
^{0}C	degree centigrade
2,4 DNPH	2,4 dinitro phenyl hydrazine
A-Ac	air acetylene
AAS	Atomic absorption spectrophotometer
ACP	acid phosphatase
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	Analysis of Variance
AR	analytical reagent
AST	aspartate transaminase
ATP	adenosine tri phosphate
ATPase	adenosine triphosphatase
BOD	biological oxygen demand
BTEX	benzene toluene ethyl benzene and xylene
Ca^{2+} ATPase	calcium adenosine triphosphatase
Ca^{2+}	calcium ions
CAGR	cumulative annual growth rate
CAT	catalase
CDNB	1 chloro-2, 4-dinitro benzene
CE	choline esterase

CK	creatine kinase
COD	chemical oxygen demand
Conc. $HClO_4$	concentrated perchloric acid
Conc. HNO_3	concentrated nitric acid
Cu	copper
Cyt.c	Cytochrome c
df	degrees of freedom
Dist.H_2O	distilled water
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DTNB	5, 5'- dithio-bis-2-nitro benzoic acid
ECLIA	Electro Chemiluminescence immunoassay
EDTA	ethylene diamine tetra acetic acid
EMEA	European Medicines Agency Annex
ESR	Erythrocyte sedimentation rate
eV	electron volt
F	Variance ratio
FBP	folate binding protein
$FeCl_3$	ferric chloride
$FeCl_3$-CH_3COOH	ferric chloride-acetic acid
$FeSo_4$	ferrous sulphate
FPRC	Fine packed red cells
g	gram
g/dl	gram per decilitre
g/l	gram per litre
G6PD	glucose-6-phosphate dehydrogenase
GC	Gas chromatography
GC/MS	Gas chromatography mass spectrometry
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	Total reduced glutathione
h	hour

H_2	hydrogen
H_2O_2	hydrogen peroxide
Hb	Haemoglobin
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HDL C	high density lipoprotein cholesterol
HDL	high density lipoprotein
HMG-CoA reductase :	3-hydroxy-3-methyl-glutaryl-CoA reductase
hr	hour
hrs	hours
HSA	Head space analysis
IU/L	International units per litre
K^+	potassium ions
KI	Potassium iodide
KOH	potassium hydroxide
L	litre
LC_{50}	lethal concentration causing 50% mortality
LDH	lactate dehydrogenase
LDL C	low density lipoprotein cholesterol
LERA	lysosomal enzyme release assay
LPO	lipid peroxidation
M	molar
m/z	mass to charge ratio
mA	milli ampere
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDA	malondialdehyde
MEOS	microsomal ethanol oxidation system
mg	milligram
mg/dl	milligram per decilitre
mg/g	milligram per gram

mg/l	milligram per litre
Mg^{2+} ATPase	magnesium adenosine triphosphatase
Mg^{2+}	magnesium ions
ml	millilitre
mm	millimeter
mM	millimolar
MTBE	methyl tertiary butyl ether
N	normal
Na^{+}	sodium ions
Na^{+}/ K^{+} ATPase	sodium potassium adenosine triphosphatase
Na_2HPO_4	disodium hydrogen phosphate
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	sodiumdihydrogen phosphate
NaOH	sodium hydroxide
nm	nano metre
NP-SH	Non protein sulphhydryl groups
O. mossambicus	*Oreochromis mossambicus*
O.D	optical density
PCB	parachlorobenzoate
PCV	Packed cell volume
PE	Perkin Elmer
Pi	inorganic phosphate
PLCAT	plasma lecithin cholesterol acyl transferase
POD	Peroxidase
PSH	Protein sulphhydryl groups
PUFA	polyunsaturated fatty acid
RBC	Red blood corpuscles
RNA	ribonucleic acid
ROS	reactive oxygen species

rpm	revolutions per minute
SD	standard deviation
Se	selenium
SH	sulphhydryl
Sig.	Significance level
SOD	superoxide dismutase
TAG	triacylglycerol
TCA	trichloro acetic acid
TFA	trifluoro acetic acid
TSH	Total sulphhydryl groups
U/L	units per litre
US	United States
USEPA	United States environment protection agency
v/v	volume by volume
WBC	White blood corpuscles
Zn	zinc
λ	wavelength

General Introduction

The aquatic environment being highly complex and diverse encompasses several distinct ecosystems - freshwater streams, lakes, ponds, rivers, estuaries, and marine coastal and deep ocean waters – each having unique biotic and abiotic characteristics. Aquatic environment is being polluted by man by his indiscriminate discharge of xenobiotics to meet the demands of the modern era. A xenobiotic (Greek, xenos "foreign": bios "life") is a compound that is foreign to a living organism, which on interaction with a biological system leads to various pathological states. Toxicology is a relatively new and still evolving discipline, which originates from concern for the safety, conservation and protection of aquatic environments (Butter, 1978). The term ecotoxicology, first used by Truhaut in 1969 (Truhaut, 1977) has been defined as the study of effects that chemical pollutants exert on natural biota (Kendall *et al.*, 2001). Aquatic toxicology is a branch of the science of ecotoxicology in which biochemical studies have received greater prominence.

The most used vertebrate model in ecotoxicological studies is fish. According to Vander Oost *et al.* (2003) fishes are generally considered the most feasible organisms for pollutant monitoring in aquatic system. They are able to take up and retain different xenobiotics in water via active or passive processes. The biochemical parameters of fish are sensitive in detecting potential adverse effects (Almeida *et al.*, 2002). Therefore, they can be used to detect and document xenobiotics released into their environment (Sancho *et al.*, 2003). Fish is referred to be an extremely sensitive bioindicator of aquatic pollution and is the preferred test species in toxicological screening of water (Nanda *et al.*, 2002). The unique adaptations and physiological specializations of fishes make them especially suitable for use as physiological and biomedical models. Therefore, they can be used to detect and document xenobiotics released into the aquatic environment (Sancho *et al.*, 2003).

With rapid rise in the price of crude oil and projected decrease in oil supplies, alternative fuels are receiving considerable attention (Hill *et al.*, 2006). Ethanol has recently become a widely used ingredient in reformulated gasoline. US are the world's largest fuel ethanol producer, closely followed by Brazil (Ethanol Industry Outlook, 2006). Brazil has developed the most significant program for use of ethanol as fuel in the world. With the introduction of ethanol to diesel fuel imminent, instead of MTBE (Methyl Tertiary Butyl Ether) in many states of the USA, the environmental implications associated with ethanol additive fuels need to be thoroughly investigated (Adam *et al.*, 2002). The increasing demand of alternative energy sources has created interest in using ethanol with petrol and diesel. Like oil spills, ethanol spills can have deleterious effects on aquatic environments. Point sources of ethanol in surface water bodies include accidental releases of ethanol either during transport or at industrial sites. Other sources include spills and releases in lakes and reservoirs used for recreational activity. Pure ethanol releases can occur at an ethanol manufacturing facility, along the transport system, or at the bulk terminals where it is stored and blended. However, its actual behavior in the environment is not well documented. A release of neat ethanol could degrade in a period of several days to two years. Ethanol seems to be toxic to aquatic life at high concentrations. Aquatic organisms rely on dissolved oxygen for survival. Based on chemical stoichiometry, it was estimated that 1 molecule of ethanol removes 3 molecules of oxygen from the stream. The BOD for ethanol is 1.8 grams oxygen consumed per gram of ethanol (Verschueren, 1983).

Ethanol can enter surface water through three main sources: rainwater (through atmospheric volatilization and deposition), direct discharges (from spills or motor boats) and contaminated groundwater plume migration. However, a pure product spill of ethanol or a large underground storage tank release could cause ethanol levels to become toxic to microbial and aquatic life. In ground water, ethanol levels depend upon the nature of the release and the magnitude of the release. Ethanol is lighter than water; and if released rapidly in bulk into water, ethanol will tend to remain on the surface of the water. In both running and standing surface water bodies, ethanol is not expected to volatilize quickly. Calculated half-lives of volatilization of ethanol from a model river or lake were 3.3 and 38.9 days

respectively (USEPA, 2002), and under quiescent conditions in a reservoir or lake, ethanol may persist for months in the absence of biodegradation. Biodegradation is the main method of removal of ethanol from water. It appears, however, that biodegradation will occur if water temperatures are above 10^0C, with ethanol half lives of the order of hours to days. It is thus unlikely that ethanol would persist in surface waters. The breakdown of ethanol in surface waters through biological and chemical processes could potentially result in the consumption of significant quantities of dissolved oxygen, which in turn would adversely affect aquatic life, potentially leading to fish kills. Significant spills of ethanol into the surface of water bodies that have low aeration rates (e.g. lakes, ponds and large non turbulent rivers) can cause massive killings of fish and other aquatic organisms by asphyxiation, due to the depletion of oxygen in water caused by ethanol degradation.

The presence of high concentrations of ethanol could deplete or substantially lower dissolved oxygen content in the surface water within a short period of time, potentially leading to a fish kill arising from oxygen stress. An ethanol concentration of 564mg/l in the water column causes acute toxicity to aquatic life, whereas 61mg/l in water column causes chronic toxicity to aquatic life (USEPA, 1995). Ethanol was also detected in surface water at a concentration of 4020 ppb in the Hayashida River in Japan near the site of a leather factory (Yasuhara *et al.*, 1981).

Fish kills have been documented in incidents of large release of ethanol to water bodies, generally due to the result of anoxia produced from high Biological Oxygen Demand (BOD) associated with the bacterial utilization of the alcohol. For example, in May 2000, an estimated 500,000 gallon of wild turkey bourbon comprising of 250,000 gallons of ethanol was released into the environment causing the worst fish kill in 50 to 60 years in the Kentucky River (Mead and Lander, 2000). A discharge of 2,500 barrels of beer into Clear creek near golden Colorado killed more than 50,000 fish in August 2000 (Gerhardt, 2000). A similar spill had occurred in the same location in 1991 which killed 17,000 fish.

Although ethanol itself is readily biodegraded, if it leaks into groundwater, its presence may retard the degradation of more toxic compounds, increasing the risk of groundwater contamination (European Chemicals Bureau, 2000). The presence of

ethanol in groundwater makes the scenario more complicated since ethanol can increase the plume length of the most toxic contaminants (Corseuil *et al.*, 2000). Corseuil *et al.* (2000) have stated that during an ethanol spill the highest ethanol concentration observed near the source zone was 9,600 mg/l. After 540 days, the concentration of ethanol in the experimental area was 1,740 mg/l. Even traces of ethanol were detected 14 meters from the source. Ethanol in groundwater accelerated the consumption and depletion of dissolved electron acceptors. All these demonstrate that low level of nutrients present in the groundwater reduced the rate of biodegradation of ethanol. It was also observed in the controlled release experiment with gasohol that ethanol still persisted in the aquifer 18 months after contamination. Preferential degradation of ethanol is indicated by the large increase of acetate and decrease in pH of one order of magnitude (Corseuil *et al.*, 2003). Acceptable level of ethanol in drinking water is 400µg/l. Experience has shown that due to the strong preference bacteria has for ethanol, a high concentration of ethanol in ground water would cause ground water to become anaerobic. Report of Corseuil *et al.* (2000) corroborates the view that ethanol has much longer half life in ground water. Ethanol was found in groundwater suspected of leach ate contamination at a concentration of 190 ppb (Sabel and clark, 1983).

Due to its high solubility, treatment technologies that rely on the physical separation of ethanol from water will not be effective. For example, carbon filters that are widely employed to remove other gasoline contaminants will not remove ethanol.

The toxic effects of ethanol are believed to be due to the accumulation of acetaldehyde-an intermediary metabolite of ethanol. It should be noted that acetaldehyde, the main metabolite of ethanol, possesses mutagenic potential. There is also sufficient evidence for the carcinogenicity of acetaldehyde, the major metabolite of ethanol, in experimental animals. In a recent study, there are indications that the changes to ethanol blended fuel (E 85) in the United States could increase the cancer risk due to increased emission of acetaldehyde (Jacobson, 2007).

The present work is a baseline attempt to investigate and assess the toxicity of different sub lethal concentrations of ethanol. The experimental animal selected

for the present study is a euryhaline teleost, *Oreochromis mossambicus* (Peters). It is one of the most cultivated fish species owing to their fast growing characteristics and taste and seems to be the fourth most commonly cultured food fish (FAO, 1995). It can tolerate a wide range of salinity from fresh water to waters of 30 to 48 ppt salinity (Panikkar and Thampi, 1954). Its euryhaline nature, high fecundity and growth rate etc. account for the suitability of tilapia as a culture fish. The above qualities along with its local availability throughout the year, low cost, reasonable size, its restricted niche, omnivorous feeding habit etc. make it an ideal candidate for laboratory studies.

The exposure period such as 7 and 21 days were selected as per Organization for Economic Cooperation and Development (OECD, 2000) guideline programme meant for aquatic organisms.

All investigations done during the tenure of this research work are aligned into ten working chapters in the thesis with the following objectives.

Chapter 1

1.1 To estimate the level of ethanol in an effluent using Gas chromatography/ Mass spectrometry - this study highlight the magnitude of ethanol concentration in our water bodies which had hitherto remained unknown to the scientists as well as laymen.

1.2 To determine Blood ethanol concentration detection using Gas chromatography - gives an idea about the level of ethanol present in body fluids.

Chapter 2

To elucidate the changes brought about in ion channel mechanisms - to cite the changes brought about by ethanol in membrane bound enzymes.

Chapter 3

To evaluate the haematological profile caused by ethanol toxicity - this gives an idea about the health status of the organism in relation to the various haematological parameters assayed.

Chapter 4

To study the effect of ethanol on serum trace elements using Atomic absorption spectrophotometer - to study the variations in essential elements brought about by ethanol.

Chapter 5

5.1 To evaluate the impact of ethanol on some selected biochemical parameters in serum changes in the marker enzymes were also to be studied.

5.2 Studies using Auto analyzer - to evaluate the level of stress caused by ethanol by determining the hormonal and vitamin alterations.

Chapter 6

To assess the impact of ethanol on RBC membrane stability (both *In vitro* and *In vivo*) - to measure the extent of damage brought about in RBC membrane.

Chapter 7

To assess the impact of ethanol in metabolic profiles - to study the amount of energy expended by the organism in combating the stress caused by ethanol.

7.1 Effect of ethanol on carbohydrate metabolism of fresh water fish, *Oreochromis mossambicus* (Peters).

7.2 Effect of ethanol on mitochondrial electron transport chain

7.3 Effect of ethanol on protein metabolism

7.4 Effect of ethanol on lipid metabolism

Chapter 8

To evaluate the impact of ethanol on lysosomal membrane stability (*in vitro* and *in vivo*)

Chapter 9

To study the non enzymatic and enzymatic antioxidant status caused by ethanol toxicity - to assess the detoxification capacity of the organism.

Chapter 10

To find out the gross anatomical and histopathological changes caused by ethanol in gills, liver, kidney and heart tissues - to study the pathological impact in cardinal tissues.

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Review of Literature

Contents

- Review of the toxicity and metabolic effect of ethanol
- Pathogenesis of ethanol toxicity
- Review of the methods of determination of levels of ethanol
- Review of the effect of ethanol on branchial ATPases.
- Review of the effect of ethanol on haematological parameters.
- Review of the effect of ethanol on serum trace elements
- Review of the effect of ethanol on serum parameters
- Review of the effect of ethanol on erythrocyte membrane stability
- Review of the effect of ethanol on metabolic profiles
- Review of the effect of ethanol on lysosomal membrane stability
- Review of the effect of ethanol on enzymatic and non enzymatic parameters.
- Review of histopathological effects of ethanol

Fish are the most at threat from aquatic pollution and together with their long-term exposure in natural habitat they are suitable biomonitors of environmental pollution (Padmini *et al.*, 2004). Fish is generally acknowledged as a worthy model for assessing aquatic contamination and is used as an environmental sentinel for water toxicants. Fish liver can be regarded as the body's detoxification organ and hence a target organ of various xenobiotic substances. Fish also are widely (and increasingly) used as animal models in toxicological research. Several features of fish make them valuable as models in toxicology (Ballatori and Villalobos, 2002; Hinton *et al.*, 2005; Kelly *et al.*, 1998). As vertebrates, fishes have a close evolutionary relationship to humans, with shared genes and biochemical pathways that have become even more apparent as a result of recent whole-genome analyses (Aparicio *et al.*, 2002; Jaillon *et al.*, 2004). Most of the fish species used in toxicological research are small; develop rapidly with a short generation time. Saravanan *et al.* (2009) has reported the use of employing fish as a bioindicator species in monitoring water pollution since it responds with great sensitivity to changes in the aquatic environment.

Fish models, primarily zebra fish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), are widely employed for alcohol based toxicity studies which points to the fact that fish can be used as a model organism to study the toxicity of ethanol (Yuhui Hu *et al.*, 2008). One of the potential mechanisms of ethanol toxicity

is the production of oxidative stress due to ethanol metabolism by ADH and ALDH enzymes (Dennery, 2007). Reimers *et al.* (2004) observed that oxidative stress can generate either free radicals or highly reactive aldehydes that damage macromolecules.

The effects of xenobiotic contamination in an ecosystem can be estimated through analysis of biochemical changes in organisms inhabiting that region (Brewer *et al.*, 2001; Norris *et al.*, 2000; Tuvikene *et al.*, 1996). Several specific enzymes have been proposed for monitoring purposes of water pollution (Agradi *et al.*, 2000). According to Gallagher and Di Giulio (1992) gills were the first organs to be exposed to water-borne contaminants.

Absorption, distribution, biotransformation, and excretion of xenobiotics by fish are important determinants of chemical toxicity studies. All known cellular membranes are composed of lipid bilayer arranged with hydrophilic Polar Regions facing the outer surfaces and hydrophobic regions oriented toward the interior. On either surface or traversing the entire width of the membrane are globular proteins. A primary pathway for xenobiotic transport across lipid membranes is by passive diffusion. Xenobiotics are absorbed by fish across the gills, skin, and gut. Water and blood which flow through the gills maintain xenobiotic diffusion gradients across the gill epithelium (Figure 1). The main routes of chemical uptake for fish include direct uptake from water (across gills and skin). Blood is the most accessible component of the vertebrate body fluid, to xenobiotics and has frequently been examined to assess physiological status (Houston, 1997). Elevation of metabolic rate is a common response to pollutant exposure. Biotransformation accelerates the elimination of many lipophilic compounds by converting them to more polar metabolites. The main function of biotransformation enzymes is to detoxify xenobiotics, they also convert a significant fraction to products which are more toxic, mutagenic or carcinogenic than the parent compound. Biotransformation enzymes have a wide tissue distribution, but are generally highest in tissues concerned with the ports of entry or removal of xenobiotics, which will therefore be potential sites of genotoxic effect, for example, the liver, intestines, kidneys and gills

of fish (Livingstone, 1991, 1993). Xenobiotics that percolate up to the cellular level bring about alteration in gene expression (Danzo, 1997).

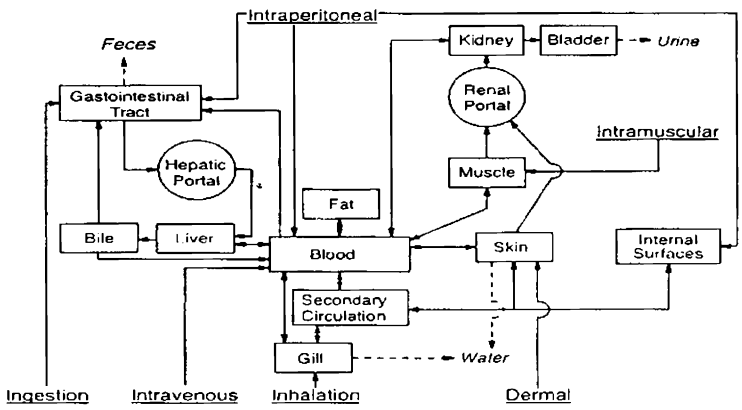


Figure1. Pathways for absorption, distribution and elimination of xenobiotic compounds in fish

Review of the toxicity and metabolic effect of ethanol.

Ethanol, also known as grain alcohol is said to be the simplest and most commonly used alcohols. Ethanol finds its wide application in paint, ink, pharmaceutical, leather and cosmetic industries. The hydrophilic nature of ethanol makes it difficult to extract it from water. Also and because of this water solubility, it readily crosses important biological membranes, such as the blood brain barrier, which in turn affect a large number of organs and biological processes in the body. Nelson and Cox (2000) have reported about the existence of ethanol in nature mainly produced by yeast and other microorganisms. Also reports given by Johnston and Bernad (1983) explained that the some vertebrate animals also produce ethanol, for example, goldfish, when exposed to low levels of dissolved oxygen in water.

There are reports suggesting that ethanol induced organ damage is caused by acetaldehyde (Tuma and Casey, 2003)

Ethanol is metabolized in the liver; this metabolized product increased the production of reactive oxygen species, which are mediators for tissue damage. Indeed, independently of necrosis and inflammation, alcohol (via acetaldehyde) can directly affect the stellate cells (also called Ito cells or lipocytes) in the liver (Lieber, 2001). 90% of ethanol is metabolized mainly in the liver.

The pharmacokinetics of ethanol determines the time course of alcohol concentration in the blood. The absorption, distribution and elimination are important in determining the pharmacodyamic of ethanol (Ramachandani *et al.*, 2001).

Liver is the main organ responsible for the metabolism of ethanol. More than 90% of alcohol is completely oxidized to acetic acid (Lieber, 1997). The rate of alcohol metabolism depends, in part, on the amount of metabolizing enzyme in the liver (Bosron *et al.*, 1993).

The hepatocyte contains three main pathways for ethanol metabolism, each located in a different sub cellular compartment (Lieber, 1997) (Figure 2).

i) Alcohol dehydrogenase (ADH) pathway of the cytosol (ii) Microsomal ethanol oxidizing system (MEOS) located in the endoplasmic reticulum (iii) Catalase located in the peroxisomes. Each of these pathways produces a specific metabolic and toxic disturbance and all three results in the production of acetaldehyde, a highly toxic metabolite (Lieber and De Carli, 1991).

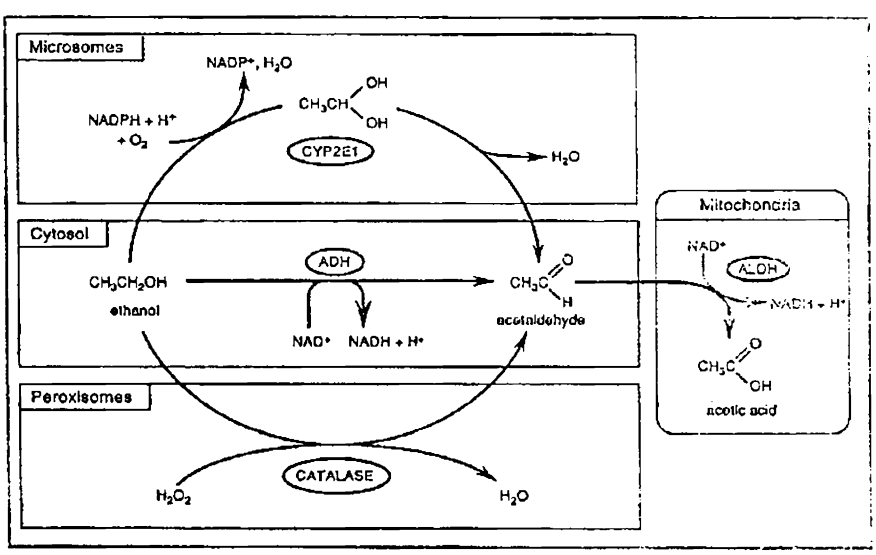


Figure 2. Pathways involved in ethanol metabolism

These pathways produce acetaldehyde which is then metabolized to acetate by aldehyde dehydrogenase.

Acetaldehyde is the most toxic metabolite of alcohol, which is thousand fold more potent reinforcer than ethanol. Acetaldehyde is subsequently metabolized to acetate through the action of aldehyde dehydrogenase. Approximately 90-95% acetaldehyde produced from ethanol oxidation is metabolized in liver mitochondria through NAD^+ dependent aldehyde dehydrogenase (ALDH), which produces acetate and consequently enters into the energy yielding processes via Kreb's cycle. One arm of the non-oxidative pathway is the synthesis of phosphatidyl ethanol (Laposata, 1997).

Global environmental benefits from using ethanol as a biofuel can also generate adverse local environmental impacts. The spoilage of the water quality and water's natural balance in its environment results in water pollution (Akman *et al.*, 2000). Donaldson (1981) explained that the indiscriminate dumping and release of agricultural, industrial and domestic wastes which contain a wide variety of organic and inorganic pollutants including solvents, oils, grease, phenols etc usually leads to environmental disturbance which is considered as a potential source of stress to biotic community. Even though Physical and chemical analysis could quantify pollutants in detail, they lack the ability to judge the impact of those on biota. Padmini *et al.* (2009) stated that any pollution either physical or chemical, changes the quality of the receiving waters.

According to global biofuel market analysis report global ethanol production is forecasted to grow at a cumulative annual growth rate (CAGR) around 6% during 2009-2018 and has reached 33,895 million gallons on amount of the high production in the US. US ethanol production base will be accredited for growth in the world ethanol production as the US accounted for 44% of the total world ethanol production in 2008. Government's plan to introduce E 85 along with the phasing out of MTBE resulted in the increased ethanol demand globally in the country (Global Biofuel Market Analysis (GBMA), 2009). Following the oil price shock in 1973, alternative fuels were studied widely and the alcohols were regarded as strong candidates to augment or even replace crude-oil based fuels. Employing pure ethanol enhances the efficiency of the fuels and also increases octane rating. These results confirmed the model's general validity and provide valuable insights relating

to the controlling role of the cool flame in the octane number determination and the use of alcohol fuels as octane blending components for use in modern and future gasoline engine technologies (Yates *et al.*, 2009). Ethanol is used as fuel in neat form in some countries (Brazil) or blended with gasoline (Europe, Canada and the United States). Fuel ethanol can be either anhydrous (containing less than 1% water which is the typical case in the US) or hydrous (containing at least 5% water by volume which is the typical case in Brazil). Brazil has developed the most significant program of ethanol use as fuel in the world.

Ethanol will enter the environment as emissions from its manufacture, use as a solvent and chemical intermediate, and release in fermentation and alcoholic beverage preparation. The largest source of ethanol release to the environment is expected to be from use of ethanol containing products, including consumer products, where applications are open and engineering controls to recover and recycle solvent are not always used. Pure ethanol releases can occur at an ethanol manufacturing facility, along the transport system, or at the bulk terminals where it is stored and blended. However, its actual behavior in the environment is not well documented. Because of the co solvency and oxygen depletion factors associated with ethanol, there is concern that a significant and continuing release (e.g. from a significant undetected UST leak) could result in an extended BTEX plume. Ethanol can be toxic to aquatic life at high concentrations. Only large concentrations (>100,000 ppm) of alcohols are generally considered to be toxic to most microorganisms, and are therefore not biodegradable (Brusseau, 1993; Hunt *et al.*, 1997a). High concentrations of ethanol may occur at fuel/water interfaces or near pure ethanol spills. Such concentrations may inhibit microbial activity near the source. Ethanol is therefore expected to travel through the subsurface at essentially the same velocity as that of water (Brusseau, 1993). Due to its high solubility, treatment technologies that rely on the physical separation of ethanol from water (e.g. adsorptive filters) will not be effective.

In general, large concentrations (10 - 15%) of alcohol are considered to be toxic to most microorganisms (Brusseau, 1993; Ingram and Buttke, 1984). Most bacteria exhibit a dose-dependent inhibition of growth over the range of 1 to 10%

ethanol by volume, and only very few species can grow at concentrations over 10% (Ingram and Buttke, 1984).

Hunt *et al.* (1997b) reported that ethanol concentrations higher than 40,000 mg/l were toxic to the microorganisms as evidenced by a complete stop in oxygen uptake. As the concentration of ethanol increased there was significant decrease in the activity of microorganisms as indicated by Araujo *et al.* (1998) Most treatment technologies that are effective in removing benzene and MTBE from water (air stripping, granular activated carbon) are not effective in removing ethanol. However, the following environmental transport properties of ethanol are cause for some concern: (1) at high concentrations, ethanol can make other gasoline constituents more soluble in groundwater (co solvency effect); (2) when present in a gasoline spill, ethanol can delay the degradation of other, more toxic components in gasoline; (3) ethanol can cause greater lateral spread of the layer of gasoline on top of the water table. The breakdown of ethanol in surface waters could potentially result in the consumption of significant quantities of dissolved oxygen in the surface water body. When BOD levels are high, dissolved oxygen levels decrease because the oxygen that is available in the water is consumed by the bacteria (Sawyer *et al.*, 2003). Depending on conditions in the surface water body and the amount of ethanol introduced, this could result in fish kills. The breakdown of ethanol in surface waters through biological and chemical processes could potentially result in the consumption of significant quantities of dissolved oxygen in the surface water body. Depending on the conditions in the surface water body and the amount of ethanol introduced, it is possible that sufficient amounts of dissolved oxygen could be consumed to adversely affect aquatic life, potentially leading to fish kills. Spills of ethanol into surface water bodies that have low aeration rates (e.g. ponds, lakes and large, non turbulent, rivers) can deplete oxygen and asphyxiate fish and other aquatic organisms.

There have been several news reports stating the spillage of ethanol resulting in fish kills. On June 21st, 2009, thousands of fish died suddenly in the Rock River, Illinois due to the derailment of the train that spilled gasoline ready ethanol into the Rock River resulting in fish kills (Figure 3). The Illinois Department of Natural

Resources (IDNR) believes that the fish kill is related to the ethanol spill that originated in Rockford (Environment News Service, 2009). Report by Kris Bevill (2009) cited that derailment of Canadian national train resulted in the leakage of an estimated amount of 55,000 gallons to 75,000 gallons of ethanol into the surrounding soils and waterways (Kris Bevill, 2009).This explains that ethanol is toxic at high concentration and can oxidize to acetaldehyde which is toxic at lower concentrations. The breakdown of ethanol in surface water consumes dissolved oxygen from the water column which can stress or kill fish and mussels (Kris Bevill, 2009).

Figure 3. Dead fish on Illinois Rock River. June 22, 2009 (Photo by W.J. Manon) (Environment News Service, 2009)

Pathogenesis of ethanol toxicity.

The consumption of alcohol affects the liver through not only by nutritional disturbances but also its direct toxicity because of its predominant metabolism in the liver associated with oxidation - reduction (redox) changes and oxidative stress. Redox changes during ethanol toxicity are generated mainly by the activity of the microsomal ethanol oxidizing system (MEOS) and its key enzyme cytochrome P450 2E1 (CYP 2E1), which releases free radicals (Lieber, 2004). Oxidative stress is characterized by excess level of ROS, abnormal lipid breakdown resulting in additional reactive molecules, and/or reduced level of antioxidants, which can eliminate reactive molecules (Figure 4).

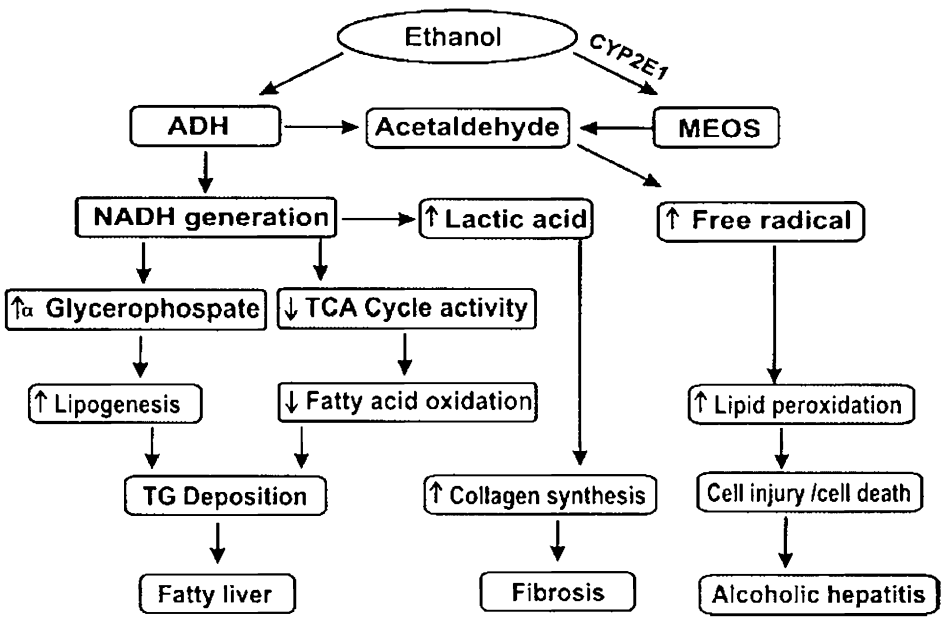


Figure 4. Pathogenesis of ethanol toxicity

A variety of antioxidant defense systems are operative, including enzymatic and non-enzymatic antioxidants (Yadav *et al.*, 1997). Enzymes directly involved in detoxification of reactive oxygen species (ROS) are superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase and small molecules such as reduced glutathione, vitamin C, vitamin E and uric acid (Revnanen *et al.*, 1998).

The body relies on several endogenous defense mechanisms to help protection against free radical induced cell damage that include the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase. SOD and CAT are the two major scavenging enzymes that remove the toxic free radicals *in vivo* (Singh, 1996). Lipid peroxidation and associated membrane damage is a key feature of alcoholic liver injury (Diluzio and Stege, 1976). Ethanol is metabolized in the liver and this organ is said to be the most affected one by ethanol toxicity (Frezza *et al.*, 1990). Ethanol oxidation causes ROS, which are mediators for the tissue damage following intoxication (Scott *et al.*, 2000). In fish G6PDH might play such a crucial role in maintaining the redox state of the cell and in modulating the antioxidant defenses through the control of NADPH generation.

Changes in the antioxidant defense system are used as biomarkers of a variety of prooxidant situations in fish, including nutritional deficiencies (Blom *et al.*, 2000; Hidalgo *et al.*, 2002 and Pascual *et al.*, 2003) and exposure to xenobiotics (Winston and Di Giulio, 1991; Pedrajas *et al.*, 1996), which can negatively affect growth, disease resistance, and behaviour. Peroxidases are a family of widespread enzymes which perform distinct tasks. On the one side they act as preventive antioxidants to detoxify damaging lipid peroxides or other peroxides from blood and organic substrates. On the other side these enzymes function as starters for oxidative reactions, thereby generating a source for reactive oxygen species. The environmental pollutants are capable of inducing oxidative stress in aquatic animal including fish. The oxidative stress is induced by the production of reactive oxygen species (ROS) (Lemaire, 1996). When the rate of ROS formation is excessive it can overwhelm the antioxidant capacity of organisms creating oxidative stress (Sies, 1993). McCarthy and Shugart (1990) suggested that oxidative stress biomarkers could be employed in environmental monitoring programmes.

Univariate techniques, particularly ANOVA, using parametric or log(x+1) transformed data, are commonly used in testing for single-species fish population endpoints, with either Dunnett's or the Student-Newman-Keuls (SNK) method serving as common post hoc tests (Graney *et al.*, 1994). When data are analyzed by ANOVA, Knauer *et al.* (2005) suggested that it might be possible to use different significance levels for abundant and for less abundant species.

Review of the methods of determination of levels of ethanol.

Kudesia (1980) explained that sugar industry and jaggery industry are the major sources of pollution brought to both aquatic and terrestrial ecosystems. The liquid wastes discharged by these industries contain large amounts of alcohols and organic compounds which seem to be lethal to aquatic flora and fauna. As reported by Salgado *et al.* (1998) usage of on-line ethanol measurements play an important role in food industry and in clinical analysis. Studies conducted by Ioffe and Vitenberg (1984) explained that static headspace gas chromatography (GC) is a technique employed for the analysis of volatile organic compounds. Deng et *al.* (2003) and Zuloaga *et al.* (2000) has stressed upon headspace analysis mainly

employed for determining the presence of ethanol in blood. Moshonas and Shaw (1994) and Nisperos-Carriedo and Shaw (1990) has found ethanol as a major compound in fresh unpasteurised orange juice by using GC/MS technique. Reports by Jorma and Ismo (1986) stated that the hypoxic state occurring in the fish on long exposure periods results in increased ethanol formation. The observations made by Rod *et al.*, (1997) has explained the presence of very low amount of ethanol mainly arised due to the presence of microorganisms in the gut region. As per the observation cited by Shoubridge and Hochachka (1980) goldfish has evolved a novel pathway of vertebrate anaerobic metabolism in which glucose carbon is metabolized to ethanol. Albert (1997) has reported that the ethanol concentration in the serum of a normal social drinker was amounted to be 1.2 mg/l. Mourik *et al.* (1982) have shown that the pyruvate dehydrogenase complex of goldfish mitochondria was able to decarboxylate pyruvate to form acetaldehyde under anaerobic conditions. Van Den Thillart (1982) has explained that the acetaldehyde thus formed is subsequently reduced to ethanol in the cytoplasm by alcohol dehydrogenase.

Review of the effect of ethanol on branchial ATPases.

Reports by Aaltonen *et al.* (2000) explained the inhibition of gill ATPases in fish species exposed to bleached kraft mill effluents. Observations by Fatima *et al.* (2000) demonstrate that paper mill effluent exposure has a deleterious membrane-damaging effect, specifically on gills. In Cichlids, post-translational modification of the Na^+/K^+ ATPase (via reversible protein phosphorylation) was suggested to be responsible for the observed decrease in their activity (Richards *et al.*, 2007). Suhel *et al.*,(2006) has observed a decrease in total ATPase as well as Na^+/ K^+ ATPase activities in the gills of freshwater fish *Channa punctatus* (Bloch) when exposed to a diluted paper mill effluent for 15, 30 and 60 days. Haya *et al.* (1983) reported that environmental organic pollutants usually affect the Na^+/K^+ ATPase by decreasing its activity. Inhibition of Mg^{2+}ATPase activity by paper mill effluent may reduce ATP production as this enzyme has been reported to be involved in oxidative phosphorylation (Racker *et al.*, 1975). Evans (1987) opined that gill ATPases are useful nonspecific biomarker in environmental stress conditions. Stagg *et al.* (1992a)

has explained that inhibition of the enzyme occurs before gross osmoregulatory dysfunction, which would point the use of Na^+/K^+ ATPase activity as an early warning of pollutant induced damage to the ionic and osmoregulatory system. Thaker *et al.* (1996) explained the mechanism of the inhibition in the gill of coastal teleost *Periophthalmus dipes* exposed to chromium. Ramon *et al.*, (2002) also observed decrease in Na^+/K^+ ATPase activity under acute ethanol intoxication in lungs and kidneys of rats. Simkiss (1996) has stated the inhibition of gill (Na^+/K^+) ATPase brought about by river pollutants that have slightly impaired one of the main biochemical systems of fish involved in their ionic regulation. Oliveira *et al.* (1996) explained that the severity of gill damage depends on the concentration of toxicants and on the time of exposure. An acute injection of ethanol was shown to inhibit Ca^{2+}ATPase activity (Ross *et al.*, 1985). Similar decrease in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase were observed in the ethanol treated group than the control rats when subjected to 30 days of toxicity studies (Arvindkumar preeti *et al.*, 2008). Rahman *et al.* (2000) reported ATPase activity as meaningful indicator of cellular activity which forms a useful toxicological tool.

Review of the effect of ethanol on haematological parameters.

James *et al.* (1998) observed a decrease in Hb, RBC count, haematocrit as well as increase in erythrocyte sedimentation rate and WBC count was observed in *Oreochromis mossambicus* when exposed to copper. Kalpana *et al.* (2009) reported an increase in haemoglobin, haematocrit, RBC and WBC in rats when subjected to sub chronic toxicity studies using cobalt. Lee and Becker (1989) stated that ethanol causes several haemolytic disorders due to both direct and indirect effects. Tyulina *et al.* (2000) reported that, the ethanol metabolite acetaldehyde inside the erythrocyte has the ability to generate free radical species which brings about deleterious effects in erythrocytes.

Review of the effect of ethanol on serum trace elements.

Tomas *et al.* (2001) explained that trace elements such as selenium, zinc and copper are components of proteins, enzymes and antioxidants which seem to occupy a prominent role in biological systems. As per the observations of Taskapan *et al.*

(2006) trace elements such as copper, zinc and selenium were recognized as essential mediators required for the development and progression of ethanol induced diseases. Morgan (1980) observed a decrease in the serum copper levels in the hair of alcoholic patients. Taskapan *et al.* (2006) reported an increase in serum copper level in cardiomyopathic patients which mainly relates to the oxidative damages or inflammation arising due to stress condition. As per the observations of Morgan (1980) increase in serum copper level is directly related to ethanol itself (as anorexia, malabsorption, or enhanced oxygen consumption resulting from activation of the microsomal ethanol-oxidizing system), ethanol-related illness (as chronic pancreatitis or cirrhosis). Alcohol is known to have an adverse effect on the status of zinc resulting with hypozincemia condition, much noted in alcoholic patients (Gordon *et al.*, 1987). Oster (1993) reported an increase in serum zinc levels mainly arising due to hypercholesterolemic condition in patients with coronary heart disease. The decrease in serum zinc concentration was observed after stress and trauma, and in several malignancies as reported by Gupta *et al.* (1993) and Gaetke *et al.* (1997). Foote and Delves (1984) explained deficiency in zinc arising mainly from alcoholism and liver cirrhosis. Decreased concentrations of serum zinc can be connected to the disturbed immune system of chronic alcoholics with cerebral dysfunction as mentioned by Menzano and Carlen (1994). As reported by Riggio (1982) zinc deficiency has been associated with impaired glucose metabolism. Nordmann (1994) observed a decrease in selenium level in the human blood plasma which mainly arise due to excessive alcohol intake.

Review of the effect of ethanol on serum parameters.

Glucose, triglycerides, cholesterol, urea, uric acid and creatinine are referred to as the major degradation products and indicators of carbohydrate, lipid and protein metabolism (Kaplan *et al.*, 1988). As per the observations of Gill *et al.* (1991) a decrease in total serum protein has been reported in the freshwater teleost *Barbus conchonius* following endosulfan exposure. Nassr and Abdel (2007) has reported increase in serum AST, ALT in *Oreochromis aureus* when subjected to phenol exposure. Reports by Sanchez *et al.* (1988) and Mendelson and Jiner (1994) explained that ethanol exposure usually leads to an increase in the concentration of

free iron. Eisenstein and Harper (1991) explained that decrease in total serum protein is related to decrease in body weight which simultaneously brings about protein deficiency arising mainly due to protein malnutrition. Prat *et al.* (1999) observed increased serum AST activity in aquatic animals when exposed to polluted water. Schmidt and Schmidt (1963) reported that elevated serum level of AST and ALT may be due to leakage of the enzymes from the liver which indicates impaired liver function. Damage to the liver, kidney and gills was evident from the elevated transaminase activities as reported by Bernet *et al.* (2001). Kurup *et al.* (1991) reported an increase in creatine kinase in the serum of rats when treated with ethanol. Sullivan and Herbert (1964) were among the first investigators to recognize that ethanol has an effect on folic acid status. As per the reports of Charlton *et al.* (1964) and Celada *et al.* (1979) an increase in serum concentration of iron arising from increased iron absorption, often seen in people consuming large amounts of ethanol is been related to deficiency in folic acid. Ethanol decreases the enterohepatic circulation of folate on acute toxicity studies as reported by Forman (1988). Baraona *et al.* (1977) observed increase in serum ferritin on prolonged exposure to alcohol. Chronic ethanol abuse often leads to the development of vitamin deficiencies, with folate deficiency being one of the most common as reported by Eichner and Hillman (1971).

Review of the effect of ethanol on erythrocyte membrane stability.

Chiu and Lubin (1989) explained that abnormalities resulting in RBC deformability and membrane permeability have been identified as defects in cellular properties that had contributed to RBC senescence. According to Lubin and Chiu (1982) oxidative damage alters the membrane permeability which leads to hemolysis. Sozmen *et al.* (1994) reported that alcohol causes membrane deformity, and brings about modification in the osmotic fragility of different cell types. Cunha *et al.* (2007) has stated that erythrocytes suffer lysis depending upon the concentrations of ethanol. As reported by Chi *et al.* (1990) and Prokopieva *et al.* (2000), ethanol exhibits a denaturating effect on erythrocyte membrane bringing about abnormal RBC morphology which results in an increased susceptibility to

hemolysis. Ivanov (2001) stated that the increase in RBC hemolysis observed can be due to the increased activities of serum specific enzymes.

Earlier studies relating to alcohol intoxication in humans and some other animals clearly indicate that ethanol affects the physicochemical properties of the cell membrane (Aufrere *et al.*, 1988; Beauge *et al.*, 1988 and Stibler *et al.*, 1991). Owen *et al.* (1982) and Sun and Sun (1983) reported membrane fluidity as influenced chiefly by lipids as an essential property of alcohol intoxication. Trandum *et al.* (1999) and Wirkner *et al.* (1999) explained that unmetabolized ethanol brings about direct effects on cells, which in turn has shown that it can permeate through membranes and can cause disruption of normal cell structure and metabolism. Increased Cholesterol/Phospholipid ratio in alcoholic erythrocyte membrane indicates decreased fluidity which influences viscoelastic properties of the membrane which seems to be in agreement with the reports cited by Beauge *et al.* (1985), (1988) and Stibler *et al.* (1991). Dumas *et al.* (1997) indicated that cholesterol incorporation in the hydrocarbon core of erythrocyte membrane enhances oxygen diffusiveness.

Review of the effect of ethanol on metabolic profiles

Das *et al.* (2004a) reported cytoplasmic enzyme LDH as the most widely used marker of organ or tissue lesions in toxicology and in clinical chemistry. LDH has also been used as an indicator of hypoxic conditions in the organism (Das *et al.*, 2004a). Reports made by David and Ray (1966) explained that industrial effluents when discharged into aquatic ecosystems cause oxygen depletion and induce high fish mortality by interfering with the respiratory metabolism. Sadakat Ozdil *et al.* (2004) observed an increase in serum LDH in rats when treated with ethanol. Khadiga *et al.* (2002) also reported an increase in serum LDH in *Oreochromis niloticus* (L.) in response to ambient water pollution. Fernandes *et al.* (2008) has reported an increase in blood glucose levels in mullets which revealed the presence of contaminants. Kalapana *et al.* (2009) observed an increase in blood glucose in the male Sprague-Dawley rats when treated with cobalt chloride for 7 days. Ellis (1937) stated that pollutants affect fish mainly in three ways such as (i) by causing a

respiratory and circulatory failure by interfering with excretory functions of the gills, (ii) by specific toxic action after being absorbed through gills, oral mucosa, and other external structures and (iii) by toxic action after being absorbed through the gastro-intestinal tract. Observations made by Clarke (1975) points out that carbohydrate is considered to be the first among the organic nutrients which is been degraded in response to stress conditions imposed on animals. Wong- Riley (1989) reported that azide brings about inhibition in cytochrome oxidase which is referred to as a rate-limiting enzyme in oxidative phosphorylation. Cunningham *et al.* (1990) and Cahill and Cunningham (2000) observed that chronic ethanol treatment affects mitochondrial oxidative phosphorylation in the liver thereby suppressing the synthesis of protein subunits which has been encoded on mitochondrial DNA (mt DNA). Partridge *et al.* (1994) reported that inhibition of cytochrome oxidase increases reactive oxygen species. Reports by Oikari and Nitylla (1985), Vijayram *et al.* (1989); Vijayam *et al.,* (1991) Chandravathy and Reddy (1994) and recently by Sivaramakrishna and Radhakrishnaiah (1998) and Baruah *et al.* (1998) studied on the effect of aquatic pollutant on physiological and biochemical functioning in fishes. Fujiva (1961) described the biochemical changes occurring in the tissues of fish held in live boxes near a pulp and paper mill outface. Das and Vasudevan (2005) also observed increase in serum uric acid level, decrease in serum urea as well as increase in serum creatinine in the alcoholics. Adeyemi *et al.* (2009) reported an increase in serum ALP level in albino rats when they consumed leachate-contaminated groundwater over a period of 65 days. As per the report given by Adeyemi *et al.* (2009) damage brought to the tissues including liver, kidney, colon and red blood cells results in a moderate increase in serum level of ACP. Reports by Das *et al.* (2003) and Annoni *et al.* (1991) demonstrated hypoalbuminemia in chronic alcoholic liver diseases. A significant rise in serum creatinine concentration in fish caught from heavily and moderately polluted areas might be induced by glomerular insufficiency, increased muscle tissue catabolism or the impairment of carbohydrate metabolism (Murray *et al.*, 1990). Forman (1988) reported that ethanol inhibits protein synthesis, and results in a fatty liver and elevations in serum triglyceride levels. Tilak *et al.* (2009) observed a decrease in tissue total protein in *channa punctatus* (Bloch) when exposed to alachlor, a chloroacetanilide herbicide

for 10 days. Remla *et al.* (1991) reported that administration of ethanol to rats causes changes in the metabolism of serum and tissue lipids. Vijayammal and Ashakumary (1993) and Rajasree *et al.* (1999) observed a decrease in HMG CoA Reductase activity in the tissues of rats treated with alcohol under chronic exposure period. Krajnovic-Ozretic and Krajnovic-Ozretic (1992) reported that the rise in triglycerides could be an indicator of altered fat metabolism in the liver. As per the observations of Indira *et al.* (2001) a decrease in serum HDL-cholesterol followed by an increase in LDL+VLDL-cholesterol was found in rats treated with ethanol for 15 days. Atef (2007) has also observed an increase in serum cholesterol and serum lipase in teleost fish *Oreochromis niloticus* subjected to different sub lethal concentrations of nickel exposure for three weeks. Murthy *et al.* (1994) cited marked alterations in lipid metabolism in chronic alcoholics which is mainly due to their active mobilization towards the blood and/or tissue metabolism. Lieber and Baraona (1979) reported the changes in serum lipids as a sensitive indicator of the progression of liver damage in the alcoholics. Bindu and Annamalai (2003) observed an increase in lipid profiles such as total cholesterol, triglycerides, LDL-cholesterol and reduction in the HDL-cholesterol in the serum of rats when being treated with ethanol for 30 days. Harpert *et al.* (1977) reported the reduction of lipid content arising due to the utilization of lipids for energy demand under stress condition.

Review of the effect of ethanol on lysosomal membrane stability.

According to Galloway *et al.* (2004); Moore, (2002) and Moore and Noble (2004) lysosomal perturbations have been widely used as early indicators of adverse effect brought about by the pollutants exposure. Furthermore the reports by Dayeh *et al.* (2004); Galloway *et al.* (2004); Hankard *et al.* (2004); Moore and Stebbing, (1976); Martinez *et al.* (2005); Svendsen *et al.* (2004) and Servais *et al.* (2005) explained the fact that lysosomal membrane stability has been a generic indicator of cellular health in eukaryotic cells, as indicated by the studies with protozoans, coelenterates, annelids, crustaceans, molluscs, fish and mammals. The findings cited by Krishna Kumar *et al.* (1994) and Lowe *et al.* (1981) indicated that lysosomal parameters are all strongly correlated, which supports the hypothesis that stress or

pollution induced lysosomal injury is mechanistically linked with increased autophagy, lysosomal hydrolase activity, consequent cell degradation and organ atrophy. Allen and Moore (2004) observed lysosomal stability as a good indicator of physiological fitness in fish liver. Lysosomal damage is well-established as a bio-marker of stress in a wide range of vertebrates and invertebrates (Bayne *et al.*, 1976; Moore, 1990, Tabata *et al.*, 1990). Moore (1985) reported that many xenobiotics evoke alteration directly in the bounding membrane of the lysosomes. A distinct decline in the stability of lysosomal membrane in relation to contaminant burden has been reported by Kohler *et al.* (1986), Kohler (1989a, 1990) and Ward (1990). Ulf *et al.* (2001) and Zhao *et al.* (2003) pointed out that extensive release of lysosomal content results in necrosis, while partial lysosomal rupture mediates apoptosis. The studies conducted by Farnley *et al.* (2000) observed that reduced lysosomal stability can lead to impaired immunocompetence and to autophagic loss of body tissue. Krishna Kumar *et al.* (1997) observed that lysosomal membrane stability was significantly decreased when digestive tissue of *Mytilus edulis* was exposed to micro capsulated polycyclic aromatic hydrocarbons for 30 days. Brunk *et al.*, (2007) explained that during oxidative stress, large amounts of hydrogen peroxide enter the lysosomal compartment resulting in the formation of abundant hydroxyl radicals, or highly reactive iron-centered radicals, both of which endanger the stability of lysosomes. Studies conducted on lysosomal stability during oxidative stress revealed that, rather than being the sturdy organelles they were considered to be, lysosomes are quite sensitive to oxidative stress, suffering membrane labilisation and rupture leading to apoptosis or necrosis (Brunk *et al.*, (2001). Vieira de silva (1969) explained that lysosomes that are broken down results in an increased level of several hydrolytic enzymes which mainly arise due to stress condition.

Review of the effect of ethanol on enzymatic and non enzymatic parameters.

Aquatic systems often act as a sink for environmental pollutants, and although much less-studied than mammalian systems, there is a growing body of literature on the oxidative stress response of fish models (Livingstone, 2001). In general, the mechanisms of oxidative stress in aquatic organisms are similar to those

in mammals (Di Giulio *et al.,* 1989; Livingstone, 2001), and studies on oxidative stress in piscine systems have shown responses qualitatively comparable to those of mammalian systems (Kelly *et al.*, 1998).

Oxidative stress is well recognized to be a key step in the pathogenesis of ethanol-associated liver injury. An imbalance between oxidants and antioxidants can lead to oxidative stress, characterized by escalating cell damage. The metabolic effects of alcohol are due both to its direct action and to that of its first metabolite acetaldehyde, and can also be connected with the changes in redox state. The ecotoxicological approach based on biomarkers measured in individuals relies on the fact that changes occur at low levels of biological organization before the community is affected. Biomarkers are biological parameters measuring behaviors, physiology, biochemistry, cell integrity, genomic structure, and expression (Vasseur and Cossu-Leguille, 2003). They are indicators of either a normal status, or changes in individual of the population studied. The enzymatic antioxidants are well documented but the use of non-enzymatic antioxidants as biomarkers, particularly in fish, is not well known except for a few studies (Ahmad *et al.*, 2000; Pandey and Pandey, 2001; Parvez *et al.*, 2003).

Padmini and Usha Rani (2009) observed increase in the levels of lipid peroxidation markers such as conjugated diene (CD), lipid hydroperoxide (LHP) and lipid peroxide (LPO) as well as decrease in the level of reduced glutathione and antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase activities in the fish hepatocytes obtained from polluted Ennore estuaries when compared to that of unpolluted Kovalam estuaries. Sawyer *et al.* (2003) reported that when BOD levels are high, dissolved oxygen levels decrease because the oxygen that is available in the water is consumed by the bacteria. Nair *et al.* (1986) demonstrated that elevation of MDA concentration is due to the increased peroxidation of lipid membranes and is an indicator of oxidative stress. Struznka *et al.* (2005) reported GSH as the major non-protein thiol in animals, comprising up to 90% of the intracellular non-protein thiol content which serves as an important non-enzymatic antioxidant, protecting cells from toxins such as free radicals. Cossu *et al.* (1997) opined that decreased GR

activity may lead to GSH depletion. One of the potential mechanisms of ethanol toxicity is the production of oxidative stress due to ethanol metabolism by ADH and ALDH enzymes (Dennery, 2007). Reimers *et al.* (2004) reported that oxidative stress can generate either free radicals or highly reactive aldehydes that damage macromolecules. For this reason, the detection of oxidative stress is mainly based on the quantification of malondialdehyde (MDA) which is formed as a result of peroxidation of polyunsaturated fatty acids (Rio *et al.*, 2005). Reports by Niemela, (2001); Nakamura *et al.* (2003) and Sun and Sun, (2001) explain that one of the important mechanisms of ethanol toxicity is lipid peroxidation through the induction of the formation of free radicals and/or acetaldehyde adducts, which could be associated with damage to brain and other organs. Valeska *et al.* (2009) observed a decrease in GPx activity in the golden mussel *Limno perna fortunei* indicating that it can be used as a biomonitor of environmental pollution in the Suquia River basin around Cordoba city (Argentina).

Review of histopathological effects of ethanol.

Padmini and Usha Rani (2009) observed vacuolation and structural disruption in the hepatocytes of grey mullet in polluted estuaries. Wolf and Wolfe (2005) and Borges *et al.* (2006) reported vacuolization, which tends to be uniformly distributed, in the livers of fishes that are exposed to toxic contaminants. Ferguson (1989) reported that toxic exposure can result in accumulations of fat or glycogen in the liver. Hinton and Lauren (1990) and Fernandes *et al.* (2008) reported liver histopathology as a biomonitoring tool that provides assessment of effects of environmental stressors on wild fish populations, which was even proposed to be one of the most reliable indicators for fish health impairment caused by anthropogenic activities. Carrola *et al.* (2009) observed focal bile duct hyperplasia, hepatocellular necrosis and hepatocellular vacuolization in the livers of fishes sampled from the polluted sites. Vandyk *et al.* (2007) observed vacuolation indicating nuclear atrophy and lipid accumulation in the liver of *Oreochromis mossambicus* (Cichlidae) on exposure to cadmium and zinc. Tilak *et al.* (2009) observed marked histopathological alterations in tissues such as gills, liver and kidney of *Channa punctatus* (Bloch) when exposed to alachlor for 10 days.

Chapter 1

1.1 QUANTITATIVE ANALYSIS OF ETHANOL IN AN EFFLUENT USING GAS CHROMATOGRAPHY/ MASS SPECTROMETRY

Contents

1.1A Introduction
1.1B Material and Methods
1.1C Results
1.1D Discussion

1.1A Introduction

The problem of environmental pollution on account of essential industrial growth is mainly arising due to the problem of disposal of industrial waste, whether solid, liquid or gas (Kamlesh *et al.*, 2007). Water pollution occurs as a result of the presence of any objectionable or waste material capable of damaging the water quality. With the global shortage of fossil energy, especially oil and natural gas, and heavy biomass energy consumption, a major focus has developed worldwide on biofuel production (Barbara, 2007). Ethanol is a major fuel additive and promising energy alternative in the future (Sasidhar *et al.*, 2007). Ethanol has already been introduced on a large scale as a fuel additive in Brazil, USA, and some European countries, and it is expected to be one of the dominating biofuels in the transport sector within the coming twenty years. Ethanol is being blended with diesel as well as with petrol. It is also used as a neat alcohol in dedicated engines, taking advantage of the higher octane number and higher heat of vaporization. Furthermore, it is regarded as an excellent fuel for future advanced flex fuel hybrid vehicles (Hahn *et al.*, 2006). Gasoline and diesel which are currently being blended with ethanol contaminates the environment during spills. The presence of gasoline, diesel and ethanol together brings about deleterious impacts on living organisms present in soil and water. The presence of ethanol delays the degradation of gasoline

present in the aquifer. Spills and leakage of pure ethanol from storage tanks can create toxicity upto 150% more than a spill from non ethanol fuel. Ethanol dissolves oxide scale from the walls of pipes and tanks, subjecting the system to internal corrosion, which leads to leaks. In the United States, ethanol constitutes 99% of all biofuels (Farrell *et al.*, 2006). Fermenting and distilling corn ethanol requires large amounts of water. Thus, a total of about 12 l of wastewater must be removed per liter of ethanol produced. More than 1,700 gallons of water is required to produce one gallon of ethanol (Pimentel and Patzek, 2008). The production and usage of ethanol contributes to pollution of air, soil, water and global warming (Pimentel *et al.*, 2008). Ethanol rapidly biodegrades in water which lowers the amount of dissolved oxygen in an aquatic system resulting in fish kills. Further ethanol is toxic to fish and other aquatic and terrestrial organisms at high concentrations. Fuel ethanol being heavier than gasoline is completely miscible in water, which will separate ethanol from gasoline at high concentrations of water. Ethanol has also got the potential to transport the components of gasoline through ground water and surface water and to spread the floating product to a larger area due to the solvency of ethanol with gasoline components and water. Ethanol when released into water will volatilize and rapidly biodegrade. The potential decrease in dissolved oxygen as a result of ethanol degradation can upset microbial functions as well as aquatic systems. Biodegradation of ethanol can decrease the dissolved oxygen in surface waters resulting in fish kills. The presence of ethanol in water lowers the dissolved oxygen levels and results in increased chemical oxygen demand (COD) and biological oxygen demand (BOD). Emergency Planning and Community Right Know Act (EPCKA) has considered waste ethanol as a hazardous chemical (USEPA, 2008). Sugar industry and jaggery industry are the major problematic sources of pollution to both aquatic and terrestrial ecosystems. The liquid wastes discharged by these industries contain large amounts of alcohols and organic compounds which seem to be lethal to aquatic flora and fauna (Kudesia, 1980). During processing, a large amount of freshwater is used by the mills which accordingly produce a huge amount of the effluent. These untreated or partially treated effluents are discharged in surface water bodies or on land. Oxidation of biodegradable organic component of the effluent results in the depletion of dissolved

oxygen in water, destroying the aquatic ecosystem (Kaushik *et al.*, 1997). Quantitative determination of ethanol and other primary alcohols is often a challenge in the food and microbiological industry, clinical studies, and forensic science. The usage of on-line ethanol measurements plays an important role in food industry and in clinical analysis (Salgado *et al.*, 1998). The available techniques of ethanol detection include gas chromatography, electrochemical and enzymatic assays. A novel method for the determination of ethanol in jaggery effluent has been developed using GC/MS. Optimization of the mass spectrometer parameters and mobile-phase composition was performed to maximize the sensitivity and reproducibility of the method. This sensitive and rapid method does not require any sample preconcentration, although bioethanol production has been greatly improved by new technologies. The short chains being volatile, remain less time in the aquatic environment, but possess high toxic potential for aquatic life. GC/MS method was mainly used to determine the presence of ethanol in an effluent, since this method is considered to be rapid, accurate, sensitive and was validated according to EMEA guide line. Chromatographic analysis of GC-MS was performed by a calibration technique, employing standard ethanol. The headspace analysis method is very frequently used for the determination of trace organic solvent impurities in effluent samples, and in solid painted materials, etc. Headspace analysis (HSA) is a method that gives information on the nature or composition of liquid and solid samples based on the results of the analysis of the contacting gas phase (headspace) using various static and dynamic versions of gas extraction (Ioffe, 1981). Static headspace gas chromatography (GC) is a technique used for the analysis of volatile organic compounds (Ioffe and Vitenberg 1984). An important application of headspace analysis mainly includes the quantitative determination of trace organic substances present in drinking, natural and industrial waters, sewage (Cruwys *et al.*, 2002). It has also been employed for determining the presence of ethanol in blood (Deng *et al.*, 2003; Zuloaga *et al.*, 2000). Hence the present study was carried out to detect the presence of ethanol in jaggery effluent sample using Perkin Elmer AutoSystem XL Gas Chromatograph equipped with head space sampler. Nevertheless, preconcentration and extraction steps were necessary before the analysis by gas chromatography, due to the complexity and low concentration of the sample.

1.1 B Materials and Methods

1.1.1B Description of the Sample used for GC/MS studies: Jaggery effluent sample was collected in dark brown coloured bottles for the purpose of the investigation. It was obtained from local industries at the point of discharge. The samples were brought to the laboratory under cold conditions. The collected samples were stored at -20^{0}C until assayed (Carolyn, 1999).

1.1.2B Estimation of ethanol in jaggery effluent using an analytical technique: Gas chromatography–mass spectrometry (GC–MS).

Principle

GC–MS is based on a combination of two analytical techniques: capillary gas chromatography and mass spectrometry. This is further based on two analytical parameters: the retention time and the mass spectrum. The analyte molecules which arrive at a vacuum chamber get ionized by 70-eV electrons. The resulting ions are then focused with an electromagnetic lens system and get directed to an analyzer (quadrupole mass filter), where they get separated under the action of an electromagnetic field and get detected based on the mass-to-charge ratios (*m/z*) and get recorded as a mass spectrum. GC/MS instruments were further equipped with mass spectral libraries, which contain upto 500000 mass spectra. The components of a test sample were identified either between a library mass spectrum and the experimental mass spectrum. Mass spectrometric detection is performed in the full-scan mode (SCAN) in which, mass spectra were measured and identified using a library of mass spectra.

1.1.3B Standard Conditions of GC/MS Analysis: Instrumentation

The experiment was carried out using a Perkin Elmer AutoSystem XL Gas Chromatograph equipped with Perkin Elmer TurboMass Gold Mass Spectrometer, Perkin Elmer TurboMatrix 40 Trap Headspace Sampler and SUPELCO 24103-U SPB^{TM}-608 column (30m X 0.25 mm X 0.25µm film). A 5µL of effluent was injected under split mode.

1.1.4B Standard Conditions of GC/MS Analysis: Chromatographic Separation

The temperature of the column thermostat was programmed from an initial value of 60^0C for 20 minutes then increased to 150^0C at a rate of 25^0C/minute and kept at 150^0C for 1 minute. The flow of the gas was set at 1ml/minute. The carrier gas employed was helium.

Procedure

10 ml of pure ethanol was used for carrying out GC/MS analysis. This serves as the standard. From this 5µL of pure standard ethanol was directly injected for carrying out GC/MS analysis. This was run to confirm the retention time and spectrum. An ethanol peak as well as corresponding standard area was obtained. It was then compared with the data in the Perkin Elmer library. Similarly 5µL of the jaggery effluent sample was directly injected for GC/MS analysis, which resulted in obtaining a peak as well as area. The mass spectral data obtained were compared to the data in the Perkin Elmer library. Perkin Elmer AutoSystem XL Gas Chromatograph equipped with TurboMass Gold Mass Spectrometer and TurboMatrix 40 Trap Headspace Sampler provided excellent accuracy and precision in carrying out the analysis of effluent sample. The measurements were repeated three times.

1.1C Results

GC/MS method was used to determine the presence of ethanol in jaggery effluent. The mass spectrum of the unknown chromatographic peak is matched with the mass spectrum of a pure compound (ethanol) from the Perkin Elmer library, and the mass spectrum is quantitatively interpreted. It was found that about 0.34µl of ethanol was present in 5µL of the effluent. The chromatographic peak and corresponding mass spectrum of pure ethanol are shown in Figures 1A to 1G. Similarly Figure 2A to Figure 2G exhibits chromatographic peak and corresponding mass spectrum of jaggery effluent.

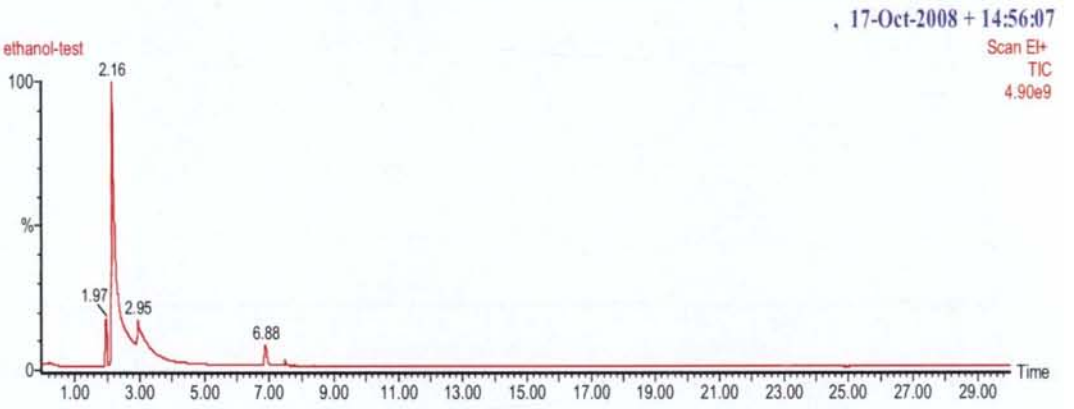


Figure 1A Standard chromatogram with solvent peak and ethanol peak

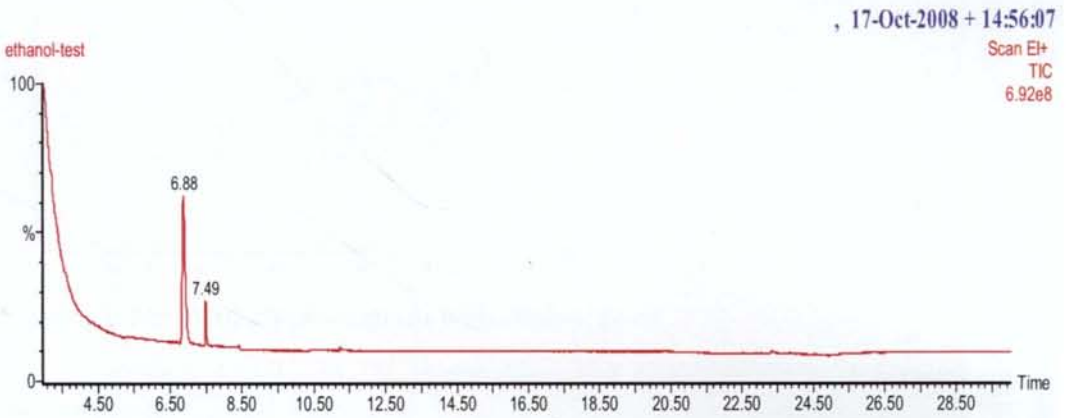


Figure 1B Standard chromatogram with ethanol peak after masking solvent peak

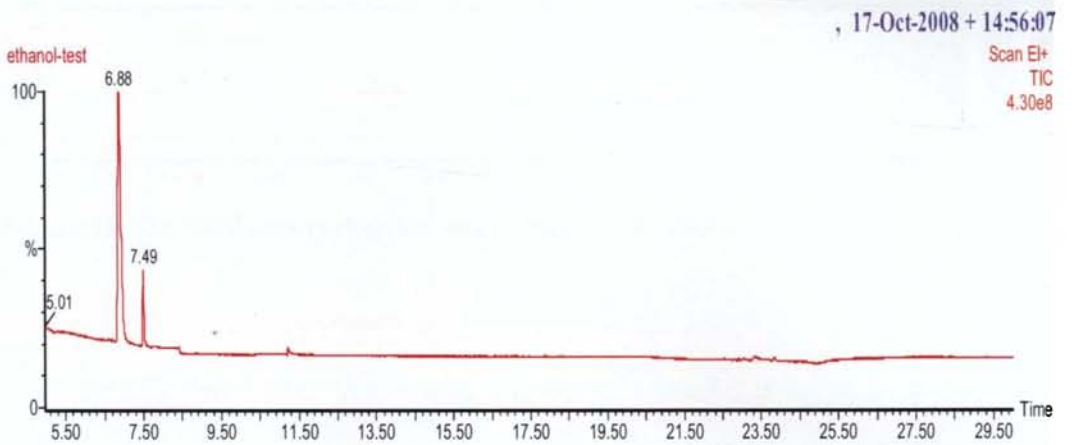


Figure 1C Standard chromatogram with ethanol peak after masking solvent peak

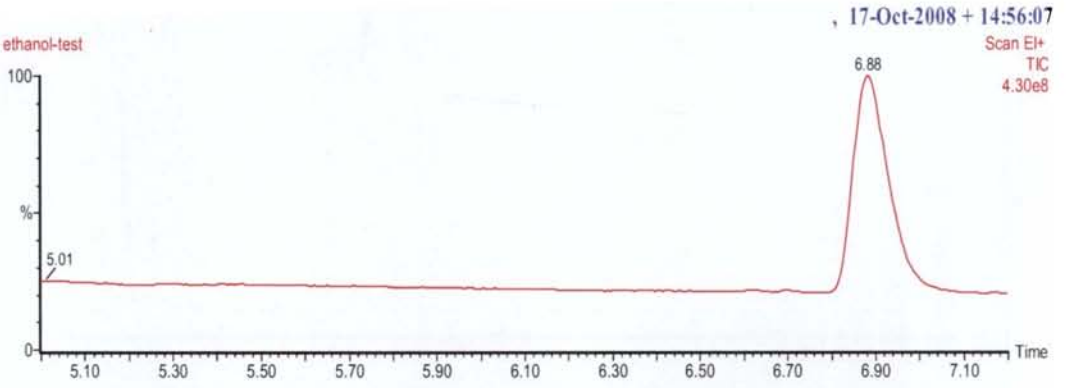


Figure 1D Standard chromatogram with ethanol peak after masking solvent peak

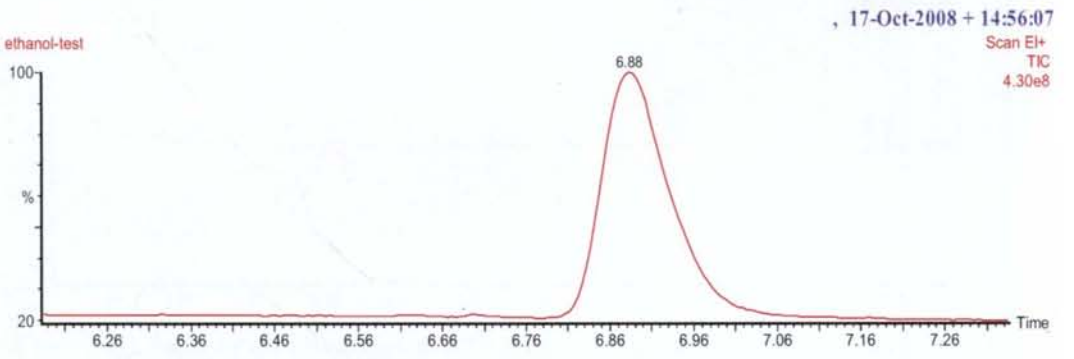


Figure 1E Standard chromatogram with ethanol peak

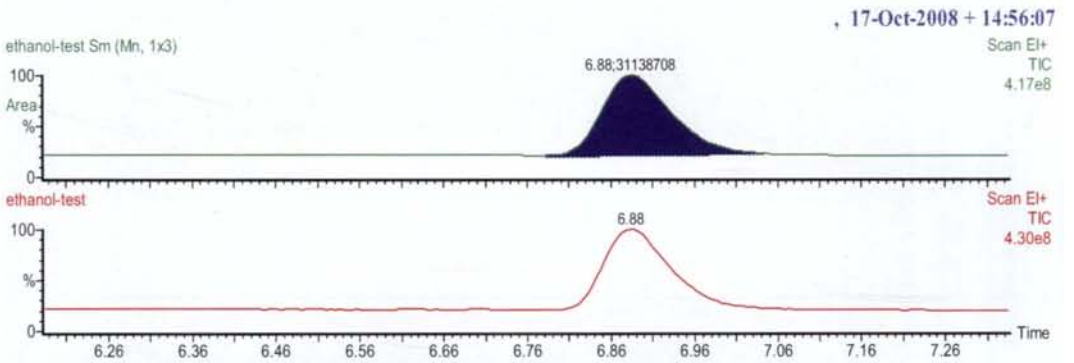


Figure 1F Standard chromatogram with ethanol peak and area (for 5µl/10ml)

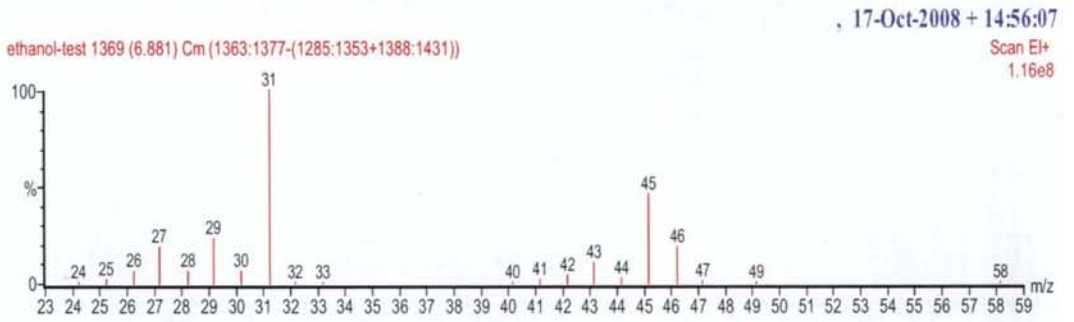


Figure 1G Mass spectrum of pure ethanol obtained from the PE library.

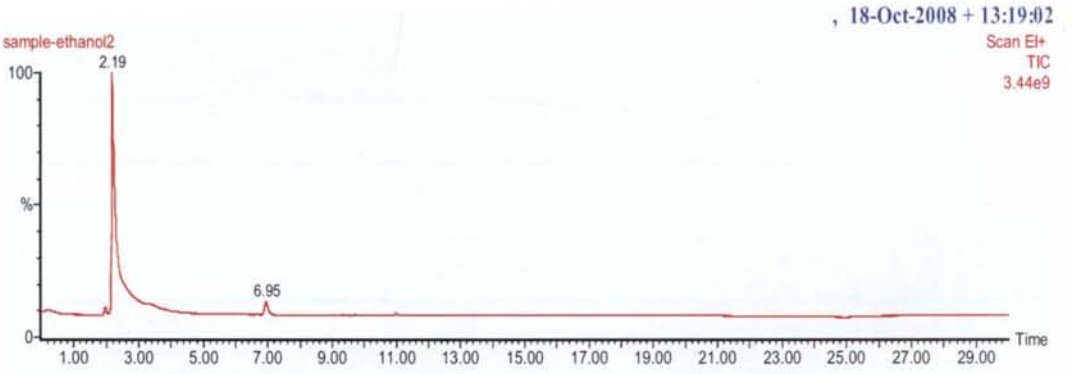


Figure 2A Sample chromatogram with solvent peak and ethanol peak

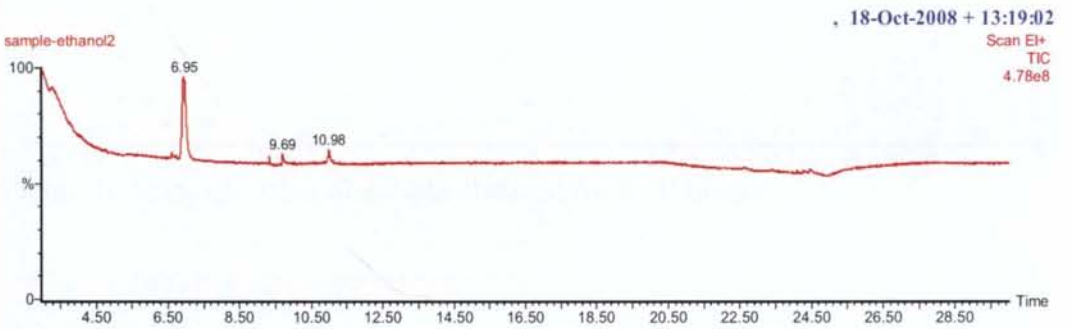


Figure 2B Sample chromatogram with ethanol peak after masking solvent peak

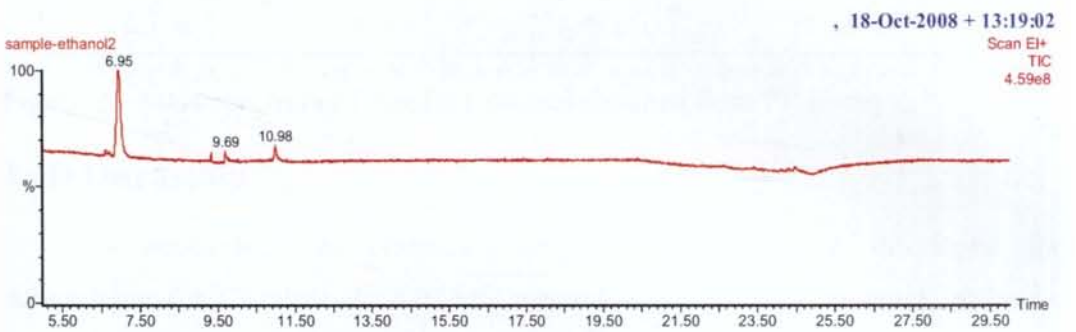


Figure 2C Sample chromatogram with ethanol peak after masking solvent peak

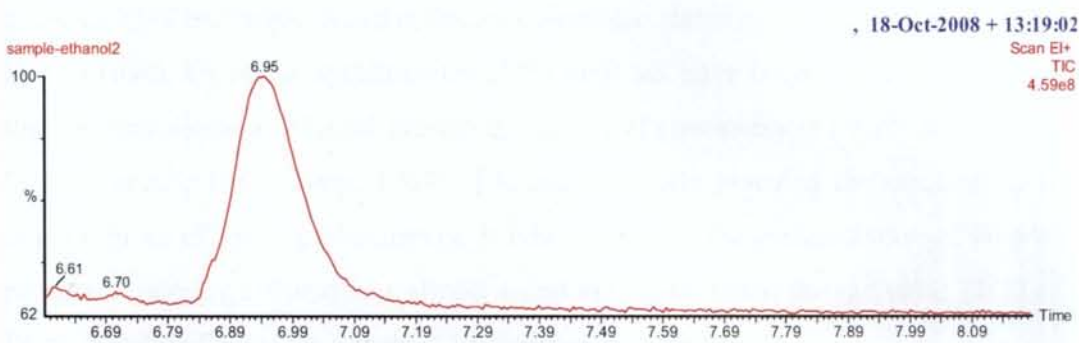


Figure 2D Sample chromatogram with ethanol peak

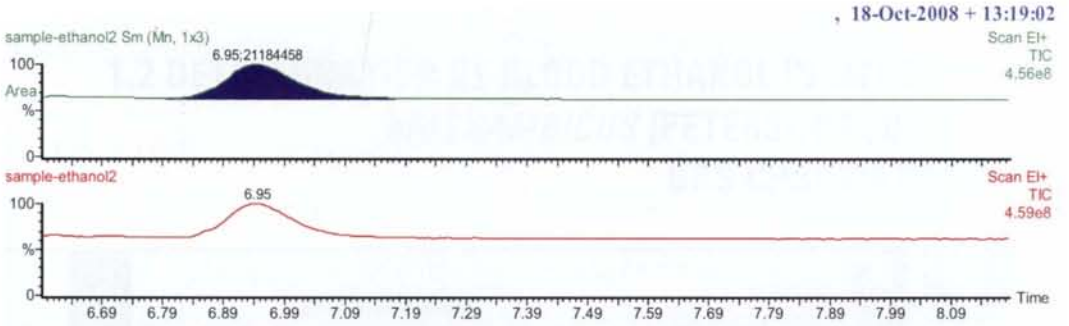


Figure 2E Sample chromatogram with ethanol peak and area

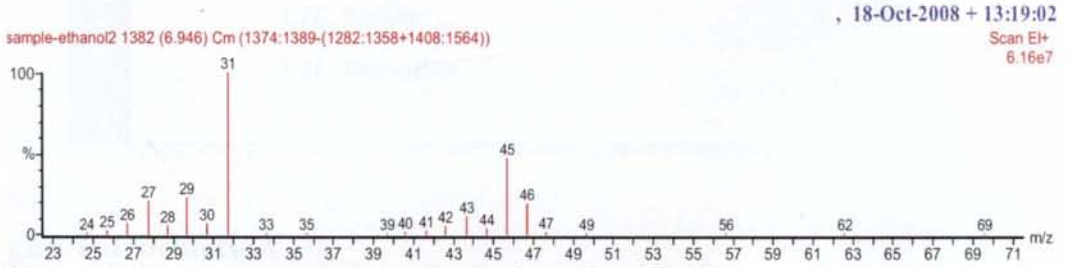


Figure 2F Mass spectrum of sample obtained from PE library

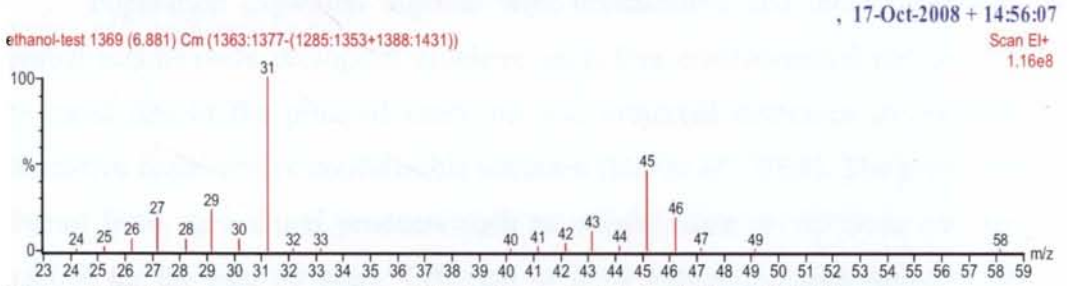


Figure 2G Mass spectrum of standard ethanol obtained from PE library

1.1D Discussion

A headspace gas chromatography method has been developed for determining C1-C3 alcohols. GC/MS method was found to be rapid, accurate, sensible and was validated according to EMEA guide line. Moshonas and Shaw (1994) has found ethanol as a major compound in fresh unpasteurised orange juice using GC/MS technique. Similar findings were also stated by Nisperos-Carriedo and Shaw (1990). GC-mass spectrometry (MS) methods have been applied to measure the concentrations of ethanol present in biological specimens (Liebich *et al*, 1977; Liebich *et al.*, 1982; Tang, 1987). The present study revealed the occurrence of ethanol in an effluent quantitatively. It was found that the concentration of ethanol present in jaggery effluent was almost equal to the sub lethal dosage value (1/20 of LC_{50}) which indicates the potential toxic impacts of ethanol.

1.2 DETERMINATION OF BLOOD ETHANOL IN *OREOCHROMIS MOSSAMBICUS* (PETERS): STUDIES USING GAS CHROMATOGRAPHY

Contents

1.2A Introduction
1.2B Material and Methods
1.2C Results
1.2D Discussion

1.2A Introduction

Population explosion together with urbanization and industrialization has created innumerable ecological problems including environmental pollution. With the rapid rise in the price of crude oil and projected decreases in oil supplies, alternative fuels receive considerable attention (Hill *et al.*, 2006). The production of ethanol from agricultural products such as starch, sugar or cellulose has been in practice for the past 80 years. Effluents of these industries cause severe pollution problems which impart the need of better waste management techniques. The effect of ethanol on zebra fish has been studied by a number of investigators, which establish the importance of fish being used as a model organism to study ethanol teratogenicity (Michael *et al.*, 2008). Gas chromatographic (GC) assays provide the greatest amount of flexibility and specificity in analyzing volatile compounds. Analysis time and resolution are the two critical factors taken into account when developing a GC assay for blood ethanol. This is based on the separation of substances on a chromatographic column, the inner surface of which is coated with a layer of a stationary phase (a viscous liquid). The process of chromatographic separation is based on various affinities of substances (sample components) to the stationary phase. The test sample components migrate along the column with the help of a carrier gas (helium); the emergence time of a substance from the column (i.e., the chromatographic retention time) is an analytical parameter. The substances

that emerge from the chromatographic column (as narrow zones) arrive at the detection system and form an analytical signal (chromatographic peak). In 1992, Tagliaro *et al.* reviewed methodologies of blood alcohol determination in which GC is referred to as the most precise and reliable method for alcohol determination in blood and other biological fluids. It has also become the gold standard in forensic toxicological studies also. In order to prevent from polluting the injection port, precolumn and column, proper dilution of the blood before analysis was carried out (Taligaro *et al.*, 1992). The objective of the present investigation was to develop a sensitive, reliable, easy-to-use, and rapid procedure for the determination of ethanol in whole blood, by using the direct injection GC technique. Direct injection and headspace GC are the two most often used GC techniques for measuring ethanol in biological specimens (Albert Tangerman, 1997).

1.2B MATERIALS AND METHODS

1.2B.1 Maintenance of Fish

Fresh water fish, *Oreochromis mossambicus* (Peters, 1852) commonly known as Tilapia was selected as the animal model for the study considering its hardy nature, ease of rearing, maintenance, availability, resistance, and economic viability. They were collected from Kerala Agricultural University, Fisheries station, Puduvyppu. They were acclimatized to the laboratory conditions for 15 days in large cement tanks filled with dechlorinated water (500L). The tanks were previously washed with potassium permanganate, to free the walls from fungal infections. The tank had a continuous and gentle flow of tap water. The physico chemical parameters of water were estimated daily according to the procedure of APHA (1998) and were maintained constant throughout the experiment. The mean values for the parameters were as follows: as dissolved oxygen of 8.16 ppm, total hardness 13 ± 2 mg/l, total alkalinity 4 ± 2 mg/l, temperature 26 ± 2^0C, pH 7.0 ± 0.33 and salinity 0 ppt. They were fed on commercial diet *ad libitum.* For experimental set up the laboratory acclimatized fish were sorted into batches of six each and were kept in 60 L tubs. Water in the tubs were changed daily. During the experimental period the animals were fed on the same commercial diet so as to avoid the effects of starvation on normal physiological processes.

1.2B.2 Determination of Lc_{50} of ethanol in *Oreochromis mossambicus* (Peters).

Lethal toxicity studies give information about the relative lethality of a toxicant. LC_{50} test was designed to determine the highest concentration of ethanol that was sufficient to kill 50 % of *Oreochromis mossambicus* and was carried out using semi static method. The median lethal concentration LC_{50} (concentration of ethanol at which 50% mortality of test population occurred) for an exposure period of 96 hr was determined by trial and error. Each experiment was repeated three times at the selected ethanol concentration, every time noting the number of fish killed at each concentration up to 96 hours. A control without the toxicant was also maintained for both lethal and sub lethal studies (Bijoy *et al.*, 2003). The average mortality in each concentration was taken to determine the LC_{50} by graphic method in which the probit mortality was plotted against log concentration of ethanol fractions by the procedure of Finney (1971). In the present investigation the effect of various concentrations of ethanol on *O. mossambicus* as a function of different exposure periods indicated that the mortality of fishes were dependent on dose and duration. Several tubs of 60 litre capacity were taken. Each tub contained 6 fishes. They were exposed to different concentrations of ethanol ranging from 1.27 g/l to 127 g/l. It was observed that at 13.01 g/l dose, 50% of the fishes were dead within 96 hrs. As per probit analysis, LC_{50} was recorded at 13.107 g/l. In this the 95% confidence limit ranged between 12.786 and 13.382 g/l.

1.2B.3 Behavioural changes observed

When fishes were exposed to different lethal concentrations of ethanol, they exhibited erratic movements, increase in opercular movement followed by a decrease, frequent surfacing and gulping, loss of equilibrium, grouping, increase in respiratory rhythm, excess secretion of mucus followed by a gradual onset of inactivity.

1.2B.4 Bioassay method

For each sub lethal experimental set up the laboratory acclimatized fishes were sorted into batches of six each and the bioassays were conducted in tubs (capacity 60 litres) static waters. For each experimentation fishes weighing 10 ± 2g

were used. To know the effect of higher concentrations as well as lower concentrations of ethanol, apart from 1.3 g/l, two more concentrations were also selected for the present study. Group 1 served as a control and was maintained in the water without addition of ethanol. Group 2 (0.65 g/l), Group3 (1.3 g/l), Group 4 (2.6 g/l) were also selected for the present studies. While in the sub lethal toxicity study, water was changed daily and the test solutions were renewed every 24 hours to maintain the dissolved oxygen concentration at optimum level (USEPA, 1975). The fishes were fed on commercial diet *ad libitum*.

1.2B.5 Experimental design for study of the effects of ethanol on immediate and prolonged exposure

For conducting experimental studies, *O. mossambicus* of 10 ± 2 g were taken in three separate tubs which contained desired concentration of ethanol (0.65 g/l, 1.3 g/l and 2.6 g/l respectively) along with tap water. Six replicates were kept for each experiment. The experimental animals were exposed for 21 days with a periodical sampling at 7 days also. During the experimental period of 21 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. Any other factor likely to influence toxicity was nullified by maintaining a suitable control.

1.2B.6 Preparation of blood sample for Gas chromatographic studies

On completion of fixed exposure periods, blood was drawn from the common cardinal vein using 1ml sterile plastic insulin syringe (Smith *et al.*, 1952) (26 mm gauge size). It was then mixed with an anticoagulant (Sodium fluoride, 2 mg/ml). The total volume was made up to 2 ml. It was then stored at 4^0C prior to analysis (Mc Carver and Durisin, 1997).

1.2B.7 Estimation of ethanol in blood using an analytical technique: Gas Chromatography (GC)

Principle

GC is a powerful and widely used technique employed for the separation, identification and quantification of components present in a mixture. In this technique, sample is converted to the vapor state and a flowing stream of carrier gas

(helium) sweeps the sample through a thermally-controlled column where the separation of components occurs. The separated components are then made to pass through a hydrogen flame detector where a complex ionization process occurs. As a consequence carbon atoms are given up in proportion to the amount of organic material present. These carbon atoms are counted as they pass through the detector and appropriate signals are transmitted to the recorder where they are transcribed in the form of a peak. Each compound that elutes from the column has a characteristic retention time, defined as the time interval from injection to peak detector response for that compound. The retention time identifies the compound; the magnitude of the detector response measures the quantity.

1.2B.8 Standard Conditions employed for GC Analysis: Instrumentation and Chromatographic separation conditions

All samples were analyzed on a GC-Varian CP 3800 gas chromatograph (GC-Varian CP 3800), equipped with a Flame ionization detector. The Flame ionization detector temperature was maintained at 150^0C. The initial column temperature was raised automatically from 60 -100^0C at a rate of 5^0C/minute. Then the final column temperature was maintained by rising the temperature from 100 to 120^0C at a rate of 20^0C/minute. The carrier gas employed was helium and its flow rate was 2ml/minute and its pressure was 1 kg/cm^2. The make up gas was nitrogen. The fuels used were of H_2/Air. The injection technique employed was of splitless mode. The sample injection volume was 1µL.

Procedure

Quantitative calculations were performed by the external calibration standard method. Five standard solutions of pure ethanol in the range of 1.57 mg/l to 7.85 mg/l corresponding to 0.2% to 1% (v/v) was taken. It was then made up to 10ml with distilled water in a standard flask. 1µL of standard ethanol was run first to confirm the retention time. When being subjected into gas chromatograph ethanol is eluted as a single peak within 5 minutes of injection. Amount of the ethanol present in the sample was calculated using standard area. The area under the peak gives an accurate representation of the concentration of ethanol present. For estimating

ethanol in blood, 1µL of the blood was directly injected into the GC injection port without any pretreatment. All the experiments were carried out on the same day. Sources of error were greatly reduced by limiting the number of steps in the protocol for the sample procedure. The area of the single ethanol peak obtained was then calculated by plotting peak area along the Y-axis and concentration along X-axis. The graph was a straight line indicating a direct proportionality. For concentration measurements, the dilution factor was taken into consideration. The results obtained were then expressed as mg/l.

1.2C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l concentrations of ethanol in the blood of *O. mossambicus* exposed for 21 days with a periodical sampling at 7 days are represented by chromatograms (Figure 1 to 3) and Tables 1.2.1 to 1.2.3 and Figure 1.2.3. Results obtained from the raw data were statistically analyzed by Two way ANOVA (Analysis of Variance) followed by Dunnett's method.

Figure 1 Gas chromatograms showing standard ethanol peak

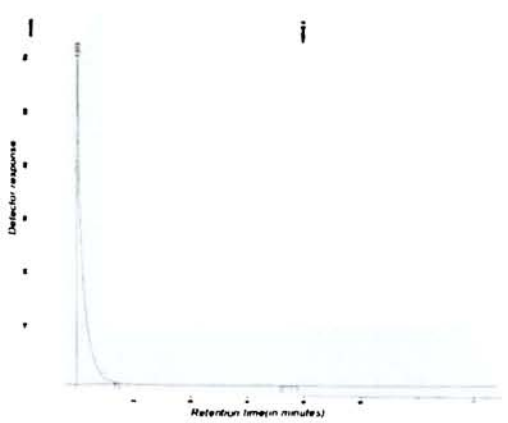


Figure.1A Gas chromatogram of standard ethanol (3.14mg/l) (0.4%)

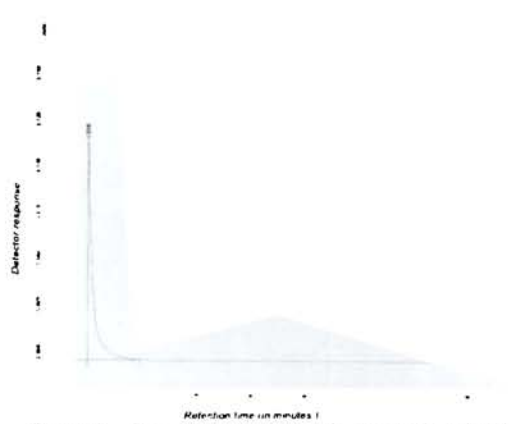


Figure.1B Gas chromatogram of standard ethanol (4.71mg/l) (0.6%)

Figure 2 Standard Gas chromatograms showing ethanol peak in the blood of *O. mossambicus* upon exposure to different sub lethal concentrations of ethanol for 7days.

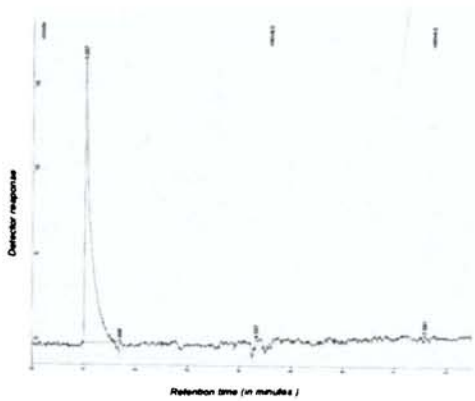


Figure.2a) Control

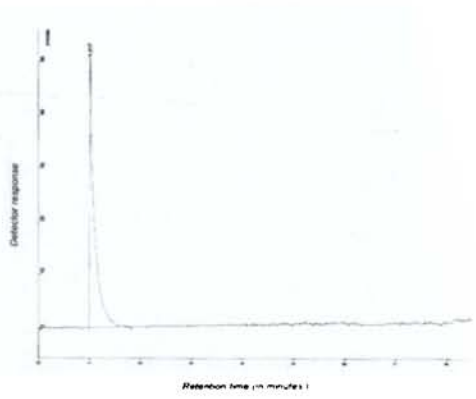


Figure.2b) 0.65g/l

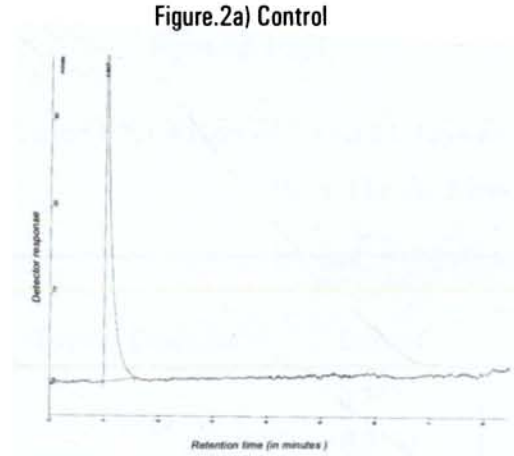


Figure.2c) 1.3g/l

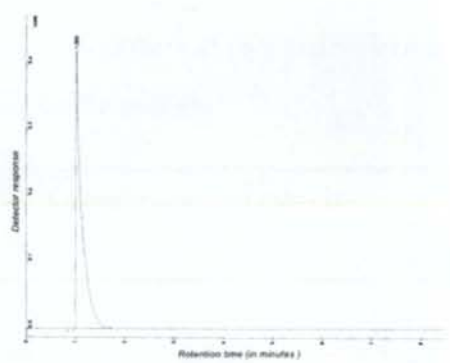


Figure.2d) 2.6g/l

Figure 3 Standard Gas chromatograms showing ethanol peak in the blood of *O. mossambicus* upon exposure to different sub lethal concentrations of ethanol for 21 days.

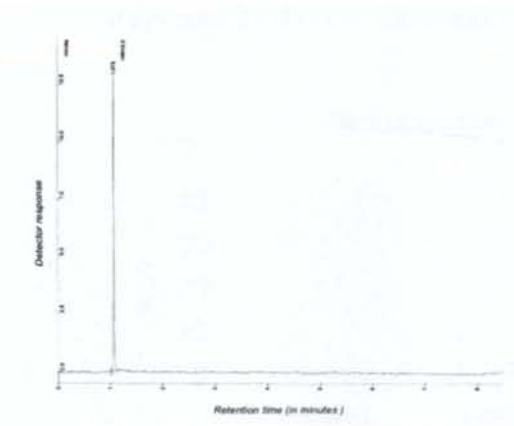


Figure.3a) Control

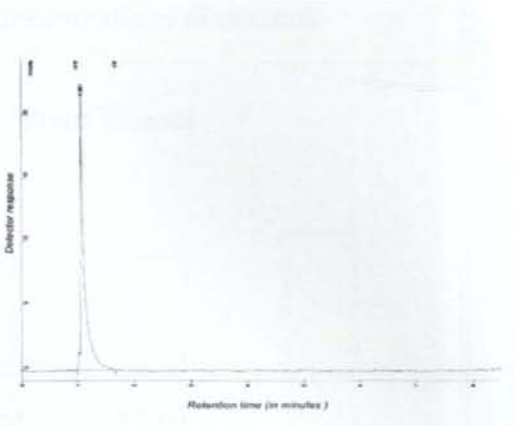


Figure.3b) 0.65g/l

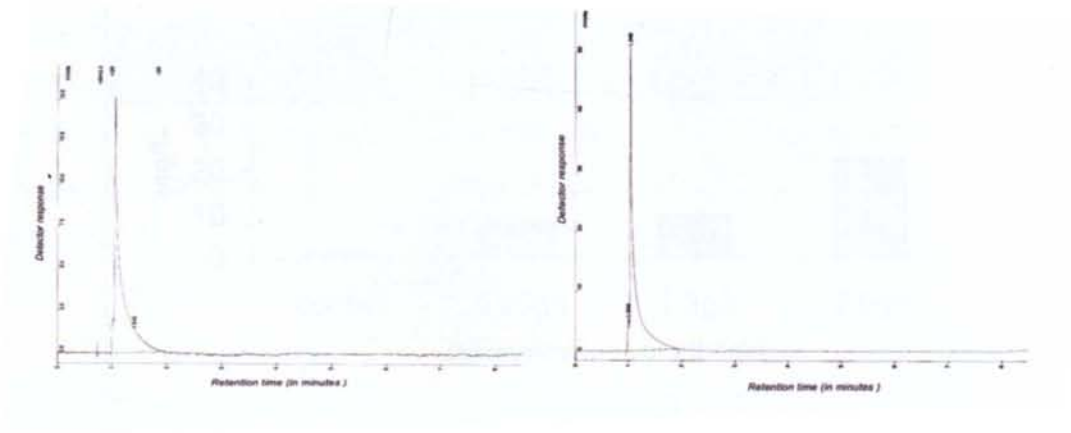


Figure.3c) 1.3g/l

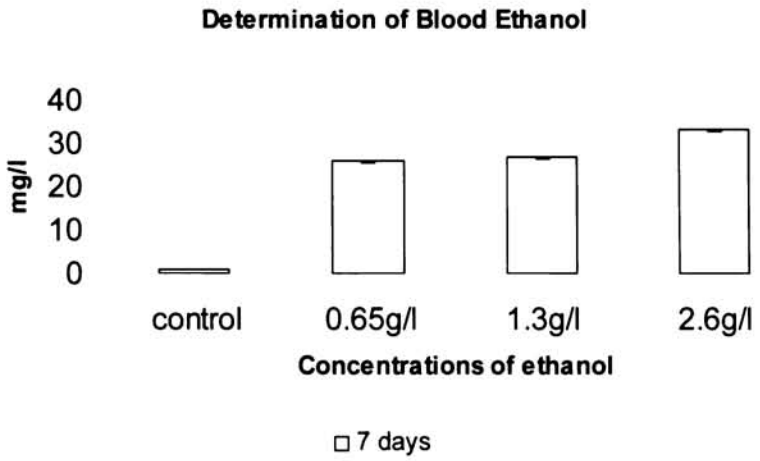
Figure.3d) 2.6g/l

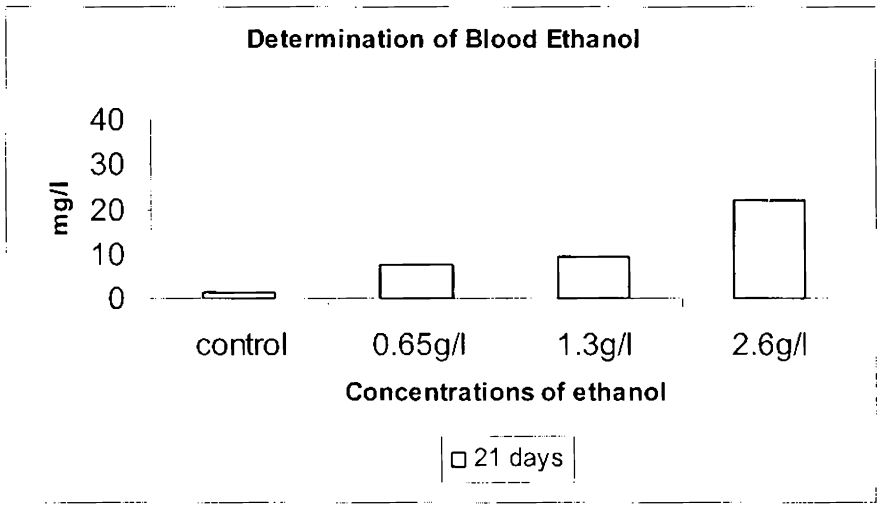
Table 1.2.1 Effect of 7 and 21 days of exposure to different concentrations of ethanol in the blood of *O. mossambicus*.

Days of Exposure	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
7 days	0.762± 0.0117	25.75± 0.1871	26.73± 0.2160	33.02± 0.1169
21 days	1.57± 0.0103	7.83± 0.0228	9.44± 0.0373	21.96± 0.0376

Average of six values in each group ± SD of six observations
Blood ethanol concentration was expressed as mg/l

Figure 1.2.2 Levels of ethanol in the blood of O. *mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant increase (P<0.001) in blood ethanol concentration was noted in *O. mossambicus* exposed to the three sub lethal concentrations of ethanol as compared to control group (Figure 1.2.3). Investigations using ANOVA substantiates the above statement and the results are shown below (Table 1.2.3a).

Table 1.2.2a ANOVA Table for Blood ethanol

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	1550.754	1	1550.754	125435.96	0.000
Between Concentrations	4283.715	3	1427.905	115499.04	0.000
Days of Exposure × Concentration	679.126	3	226.375	18310.839	0.000
Error	0.495	40	0.012		
Total	6514.090	47			

df - degrees of freedom
F variance ratio
× Interaction effect
Sig. - Significance level
0.000 indicates that the values are significant at P < 0.001

Studies conducted on blood ethanol levels exhibited a significant difference between days (P<0.001). Between concentrations there was a significant difference (P< 0.001). Considering the interaction effect of both days as well as concentrations, a significant difference (P< 0.001) was observed.

Table 1.2.3 Multiple Comparison Test

	Groups	Blood ethanol
Dunnett's	Control Vs 0.65g/l	0.000[a]
	Control Vs 1.3g/l	0.000[a]
	Control Vs 2.6g/l	0.000[a]

The values are significant at a = P < 0.001.

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. In the case of blood ethanol values, significant difference (P<0.001) was observed in all the three concentrations with respect to control which explains the high specificity.

1.2 D Discussion

The behavioural changes exhibited by *O. mossambicus* upon exposure to various lethal concentrations of ethanol points to the stimulation of peripheral nervous system which results in increased metabolic activities. The higher metabolic rate indicates that more oxygen is being utilized. Increase in opercular movement is in response to the toxicant ethanol. Observations made by Yadav *et al.* (2007) corroborates the above statement. He also found similar increase in opercular movement in *Channa striatus* exposed to fertilizer industrial waste water. The decrease in opercular movement is referred to as a unique adaptive feature to avoid intake of toxicant. Frequent surfacing and gulping shows the effort of the animal to cope with the deficiency of oxygen, and fill the two lateral highly vascular air sacs with fresh air for accessory respiration. On initial exposure to ethanol, the fish *O. mossambicus* exhibited characteristic avoidance behaviour by rapid swimming, stretching half of their body out of water surface and trying to jump out. Fish secreted copious amount of mucus, a defence mechanism to neutralize the effect of ethanol which gradually covered the entire body, gills etc. In the terminal phase of intoxication, the fish lost their balance and equilibrium and died. Rakesh *et al.* (2009) also observed similar altered behavioural changes in fresh water air breathing catfish, *Heteropneustes fossilis* (Bloch) when they were subjected to different concentrations of dimethoate for 96 hrs which also supported the present study.

Albert Tangerman (1997) explained the importance of maintaining a lower temperature of about 60^0C for ethanol based studies. According to Macchai *et al.* (1995), ethanol was stable for seven days in urine, serum, plasma and saliva when stored at 4^0C. In the present study an increase in blood ethanol concentration was found when *O. mossambicus* was subjected to 7 days of exposure to different sub lethal concentrations of ethanol. This was mainly brought about by the stress in fish when subjected to ethanol toxicity. A state of stress brought about by ethanol made *O. mossambicus* enter into a state of anoxia, which in turn increased the rate of blood alcohol concentration in *O. mossambicus*. This was mainly attributed to the enhanced activity of hepatic ADH or to increased gluconeogenesis. Observations made by Badawy (1998), Mezey (1998), Johnston and Bernard (1983), Richard *et al.* (2000) and Shoubridge and Hochachka (1980) supported the present data. When *O. mossambicus* was subjected to 21 days of exposure to different sub lethal concentrations of ethanol a significant increase ($P<0.001$) in blood ethanol concentration was observed. This is due to *O. mossambicus* entering into a state of hypoxia, resulting in the increased production of ethanol. This data suggest the ethanol production as an ubiquitous "anaerobic" end product, which accumulates whenever metabolic demand exceeds the mitochondrial oxidative potential (Milica *et al.*, 2008). Reports by Jorma and Ismo (1986) stated that the hypoxic state occurring in the fish on long exposure periods results in increased ethanol formation. This too supported the present finding. The findings made by Richard *et al.* (2000) also supports the present data. Apart from the above mentioned facts, very low amount of ethanol detected in control group of *O. mossambicus* in the present study was mainly due to the presence of microorganisms in the gut region of *O. mossambicus*. The observations made by Rod *et al.* (1997) support this finding.

An aqueous external calibrator of ethanol being used met all the requirements in which a linearity was obtained. The use of ethanol as an external calibrator was supported by Albert Tangerman (1997). Quantification of blood ethanol on the basis of peak area exhibited an excellent result. In conclusion, the direct injection method as presented here is a highly sensitive, rapid and reliable gas chromatographic technique used for measuring ethanol in blood. Once running, the method is easy to perform and does not require highly and specifically trained personnel, making this gas chromatographic method also suited to the field of clinical chemistry.

Chapter 2

EFFECT OF ETHANOL ON BRANCHIAL ATPases

Contents

2.1A Introduction
2.2 B Materials and Methods
2.2B.1 Preparation of gill sample for experimental studies
2.2B.2 Extraction of the enzyme
2.3C Results
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2.1A INTRODUCTION

Living system needs a continuous input of energy for building up and maintaining its organization. The energy rich compounds include ATP and its derivatives in a biosystem (Albert Lehninger *et al.*, 1993). ATPases represent a complex enzyme system which has requirement for cations such as Mg^{2+}, Ca^{2+}, Na^{+} and K^{+} ions for their activity. Aquatic environment easily induces stress in their inhabitants due to the variations in the quality of the water, among which industrial effluents play a major role. Fishes take up and retain different xenobiotics in water via active and passive processes. Toxicants of various origins disturb osmoregulatory potential and other physiological processes of fish (Wendelaar Bonga, 1997; Peter *et al.*, 2004).

Fish gills, which are in constant contact with the medium seem to be directly affected by contaminants. The gills of teleost fishes play an important role in ion regulation, gas exchange, acid-base balance and nitrogenous waste excretion, which means it has a key role at the interface of fish with its environment. Gill which is the main osmoregulatory surface tissue in aquatic animals seems to be the primary site

of uptake of waterborne pollutants, therefore, it is expected to be the first site where the sub lethal effects of chemicals are observed (Sancho *et al.*, 1997). Hence, several studies focus on the physiological adaptation of gills in response to fluctuating environmental scenarios (Goncalves *et al.*, 2006; Kong *et al.*, 2007a, b; Torres *et al.*, 2007). Fishes take up and retain different xenobiotics in water via active or passive processes.

Maintenance of membrane structure is essential for proper functioning of the cell. Exogenous agents like ethanol perturb cell membranes by molecular disorganization. Ethanol seems to act by altering the membrane structure and function involved in receptor and ion channel integrity. Besides, both acute and chronic administration of ethanol to experimental animals is known to impair membrane bound enzymes. There is much evidence indicating that alcohol has an important effect on biological membranes. It is also known that alcohol acts on Na^+/K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase.

Adenosine triphosphatase (ATPase, E.C. 3.6.1.3) plays an important role in supplying energy and maintaining ion concentration. ATPases are categorized among the sensitive biomarkers used for assessment of the membrane fragility of the gills (Stagg *et al.*, 1992). ATPases are the membrane-bound enzymes responsible for the transport of ions through biological membranes and thus regulate cellular volume, osmotic pressure, and membrane permeability (Sancho *et al.*, 2003). ATPases are enzymes concerned with immediate release of energy and are responsible for a large part of basic metabolic and physiological activities. ATPase activity can be taken as a meaningful indicator of cellular activity and forms a useful toxicological tool (Rahman *et al.*, 2000). Na^+/K^+ ATPase, has long been studied as a target for ethanol. Na^+/K^+ ATPase plays a pivotal role in the gills of both marine and freshwater teleosts (Evans *et al.*, 2005; Heath, 1987). This enzyme uses the chemical energy from the hydrolysis of ATP for transferring 3 Na^+ ions out of the cell and 2 K^+ ions into the cell to maintain the ionic balance. Na^+/K^+ ATPase is found in the basolateral membrane of gill epithelial cells and is involved in the active electrolyte transport across the gills at the expense of energy supplied by ATP decomposition. Mg^{2+}ATPase plays an important role in oxidative phosphorylation and ionic

transport (Gerenscer and Lee, 1983). The Mg^{2+}ATPase is used to modulate levels of the Mg^{2+} ion. The Ca^{2+} ATPase adjusts the concentration of the Ca^{2+} ion in the cytosol. The Ca^{2+}, Mg^{2+} ATPase situated in mitochondrial membranes, transfers free Ca^{2+} from the cytosol into the mitochondria only in the presence of Mg^{2+} ions (Kong *et al.*, 2004b; Zylinska and Legutko, 1998). It has been suggested that the assessment of ATPase activity may therefore be used as an early warning signal of pollutant induced damage to the osmoregulatory and acid-based regulatory system in gills (Stagg *et al.*, 1992). As branchial Na^+/K^+ ATPase is a significant osmoregulatory molecule in fish metabolism, it is important to know its natural characteristics, especially in assessing the effects of water pollution (Canli and Stagg, 1996).

The activity of ATPases in fish gill represent a useful cell-membrane biomarker of pollution as it can be easily quantified (Wendelaar Bonga, 1997). The fish gill epithelium has been extensively studied as a model for ion-transporting epithelia. An attempt has also been made to assess the possibility of using gill ATPase estimation as biomarker of exposure to ethanol in *O. mossambicus*.

2.2B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

2.2B.1 Preparation of gill sample for experimental studies

After exposing *O. mossambicus* for 7 and 21 days to different sub lethal concentrations of ethanol, gill tissues were dissected out. They were then washed in ice cold 0.33 M sucrose (pH 7.5). 10% of gill homogenate was prepared in ice cold 0.33 M sucrose. This was taken for carrying out the experimental studies.

2.2B.2 Extraction of the enzyme

Cell fractionation of the gill homogenate was carried out according to the method of Davis (1970) with slight modifications. 10% of gill homogenate was taken. It was then centrifuged at 3000g for 15 minutes in a cold refrigerated

centrifuge. Supernatant was taken. It was again centrifuged at 12000g for 30 minutes. Clear supernatant thus obtained was taken. It was then centrifuged at 35000g for 30 minutes. Supernatant so obtained was then discarded. The pellet obtained corresponds to the heavy microsomal fraction which was then resuspended in cold 0.33 M sucrose. This served as the enzyme source. One unit of ATPase activity was expressed as micromoles of inorganic phosphate (Pi) produced by ATP decomposition per milligram protein per hour.

2.2B.3 Estimation of experiments

Methods used for biochemical analysis

The following parameters were used to study ion transport mechanisms.

a. Total adenosine triphosphatase (E.C 3.6.1.3)

Principle

Total ATPase activity was estimated from the amount of Pi liberated by the method of Evans (1970). The inorganic phosphorus liberated was estimated by Fiske and Subbarow (1925) method.

Reagents

1. 0.1M Tris HCl buffer of pH 7.0
2. 0.1 M ATP
3. 0.01 M NaCl
4. 0.1M $MgCl_2$
5. 0.1 M KCl
6. 10% TCA

7. 2.5% ammonium molybdate: Dissolved 2.5 g of ammonium molybdate in 100 ml of 5 N H_2SO_4.

8. 0.25% 1,2,4 - amino naphthol sulphonic acid (ANSA): 500 mg of ANSA was dissolved in 195 ml of 15% sodium bisulphate and 5.0 ml of 20 % sodium

sulphite. Stoppered and shook until it dissolved. It was then stored in a brown bottle at 4^0C.

9. Standard phosphate solution: Dissolved 35.1 mg of KH_2PO_4 in 100 ml of water. Diluted the above solution 1 to 10 to give a working standard containing 8 µg of phosphorus/ml.

Procedure

Into a test tube, 1.5 ml of buffer, 0.1 ml each of NaCl, KCl, $MgCl_2$ and ATP was added. To This 0.1 ml of enzyme was added. The reaction mixture was incubated at 37^0C for 15 minutes. After incubation, 1.0 ml of 10% TCA was added in order to arrest the reaction. The contents were centrifuged and the supernatant solutions obtained were used for the estimation of inorganic phosphorus by the method of Fiske and Subbarow (1925).

The enzyme activity is expressed as µmoles of Pi liberated/hr/mg protein.

b. Estimation of Na^+/ K^+ adenosine triphosphatase (E.C 3.6.3.9)

Principle

Na^+/ K^+ ATPase transports Na^+/ K^+ against concentration gradient at the cost of ATP molecule liberating inorganic phosphate (Pi). Na^+/ K^+ ATPase activity was estimated from the amount of Pi liberated by the method of Bonting (1970). The inorganic phosphorus liberated was estimated by the method of Fiske and Subbarow (1925).

Reagents

1. 184 mM Tris HCl buffer of pH 7.5
2. 50 mM MgSO4
3. 50 mM KCl
4. 600 mM NaCl
5. 1 mM EDTA
6. 40 mM ATP

Procedure

To 1.0 ml of Tris buffer, 0.2 ml each of the above reagents were added. The contents were mixed together. Thus the assay medium in a final volume of 2.0 ml contained 92 mM Tris buffer, 5 mM $MgSO_4$, 60 mM NaCl, 1 mM EDTA and 4mM ATP. The tubes were then incubated for 10 minutes at 37^0C in an incubator. After 10 minutes equilibration at 37^0C in an incubator, reaction was started by the addition of 0.1 ml of 10% TCA. The contents present in the tube were centrifuged and the phosphorus content present in the supernatant was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity thus obtained was expressed as µmoles of Pi liberated/hr/mg protein.

c. Estimation of Ca^{2+} adenosine triphosphatase (E.C 3.6.3.8)

Principle

Ca^{2+} ATPase activity was estimated from the amount of Pi liberated by the method of Hjerten and Pan (1983). The inorganic phosphorus liberated was estimated by Fiske and Subbarow (1925) method.

Reagents

1. 125 mM Tris HCl buffer of pH 8.0
2. 50 mM $CaCl_2$
3. 10 mM ATP

Procedure

Into a test tube 0.1 ml each of the above reagents were added. They were then mixed well. To this 0.1 ml of the enzyme and 0.1 ml of water was added. The reaction mixture was then incubated for 15 minutes at 37^0C. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The control tubes received enzyme after the addition of 10% TCA. All the tubes were then centrifuged

and the phosphorus content present in the supernatant was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as µmoles of Pi liberated/hr/mg protein.

d. Estimation of Mg^{2+} adenosine triphosphatase (E.C 3.6.3.1)

Principle

The activity of the enzyme was estimated according to the method of Ohnishi *et al.* (1982). The inorganic phosphorus liberated was estimated by Fiske and Subbarow (1925) method.

Reagents

1. 375 mM Tris HCl buffer pH 7.6
2. 25 mM $MgCl_2$
3. 10 mM ATP

Procedure

The assay was initiated by the addition of 0.1 ml of homogenate of the gill tissue to an incubation medium which contained 0.1 ml of water and 0.1 ml of each of the above reagents. The final concentration of Tris buffer, $MgCl_2$ and ATP were 75 mM, 5 mM and 2 mM respectively in total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes of incubation at 37^0C in an incubator by the addition of 1.0 ml of 10% TCA. The tubes were centrifuged and inorganic phosphorus liberated was estimated by the method of Fiske and Subbarow (1925).

The enzyme activity was expressed as µmoles of Pi liberated/hr/mg protein.

2.3C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l of ethanol on total ATPase, Na^+/K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase activities in *O. mossambicus* are given in Table 2.3.1 to 2.3.7 and in Figure 2.3.3 to Figure 2.3.6. The results obtained on exposure to the three sub lethal concentrations of ethanol for 21 days followed by a

periodical sampling at 7 days were analyzed statistically using Two Way ANOVA of the raw data, followed by Dunnett's method.

Table 2.3.1 Effect of exposure to different concentrations of ethanol for 7 days on the gill ATPase activities in *O. mossambicus*.

Parameters Investigated	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Total ATPase	27.47±	23.87±	20.82±	16.20±
	0.2251	0.3247	0.8414	0.1102
Na^+/K^+ ATPase	12.08±	9.888±	8.392±	7.096±
	0.0143	0.1349	0.7664	0.1284
Ca^{2+} ATPase	7.728±	7.116±	6.248±	4.032±
	0.0997	0.0333	0.1573	0.4294
Mg^{2+} ATPase	7.682±	6.827±	6.201±	5.196±
	0.1070	0.1283	0.0520	0.1073

Average of six values± SD of six observations

Activities of ATPases were expressed as µmoles of inorganic phosphate (Pi) liberated/hr/mg protein

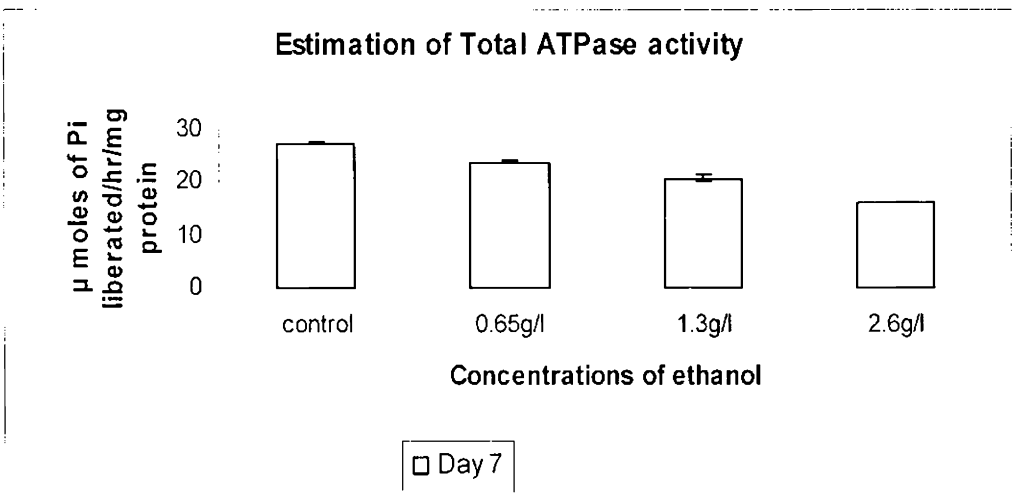
Table 2.3.2 Effect of exposure to different concentrations of ethanol for 21 days on the gill ATPase activities in *O. mossambicus*.

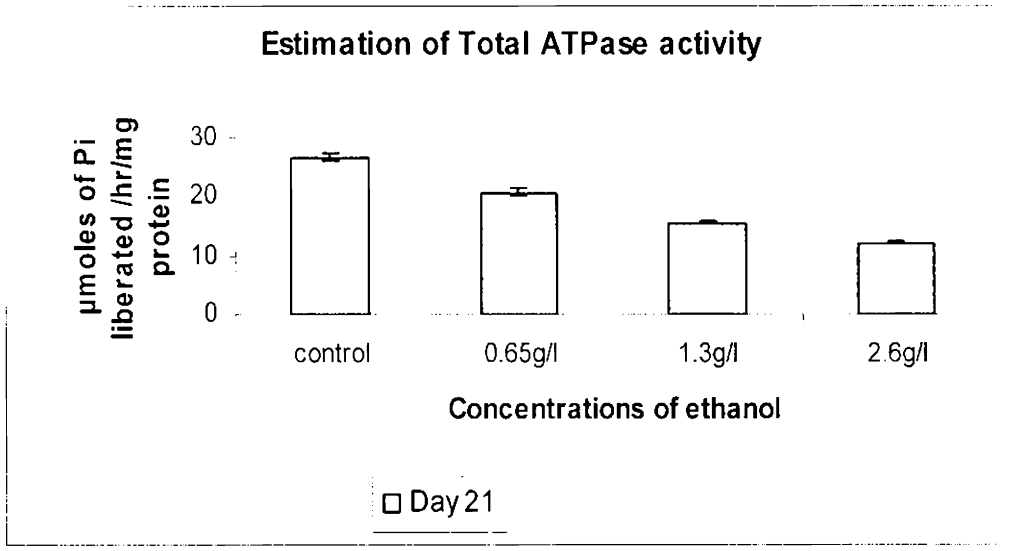
Parameters Investigated	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Total ATPase	26.64±	20.71±	15.51±	12.20±
	0.6226	0.7307	0.1586	0.0541
Na^+/K^+ ATPase	11.38±	8.960±	6.670±	5.391±
	0.1606	0.6726	0.4731	0.1692
Ca^{2+} ATPase	7.984±	4.760±	3.498±	2.675±
	0.8211	0.3814	0.3426	0.1744
Mg^{2+} ATPase	7.110±	6.932±	5.340±	4.180±
	0.0328	0.0352	0.1084	0.2537

Average of six values ± SD of six observations

Activities of ATPases were expressed as µmoles of inorganic phosphate (Pi) liberated/hr/mg protein

Figure 2.3.3 Levels of Total ATPase activity in the gill of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant decrease ($P<0.001$) in total ATPase activity was noted in the gill tissues of *O. mossambicus*, treated with various sub lethal concentrations of ethanol, with respect to control during 7 and 21 days of exposure period (Figure 2.3.3). ANOVA has been carried out to ascertain the statement and the table is shown below (Table 2.3.3a).

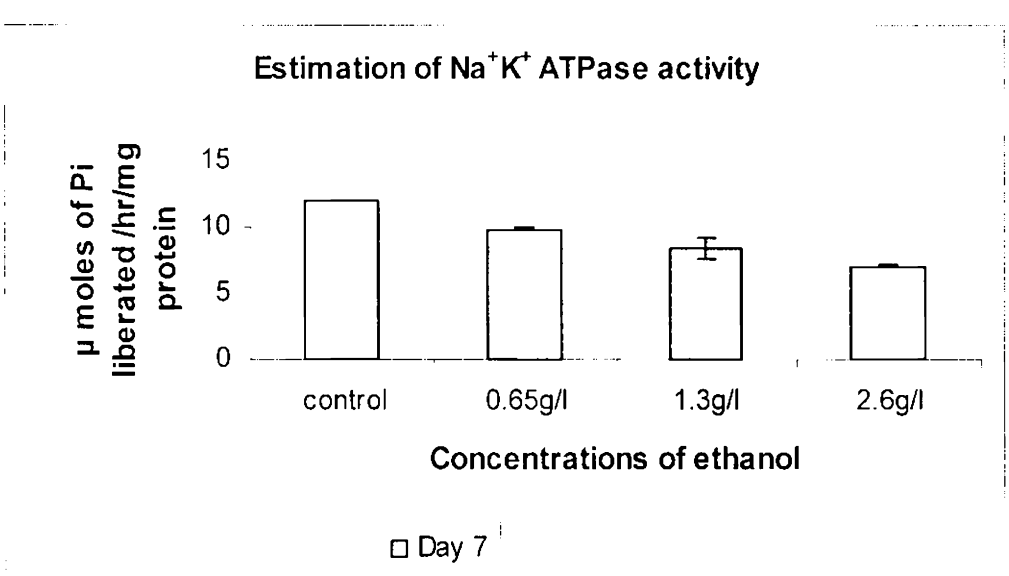
Table 2.3.3a ANOVA table for total ATPase

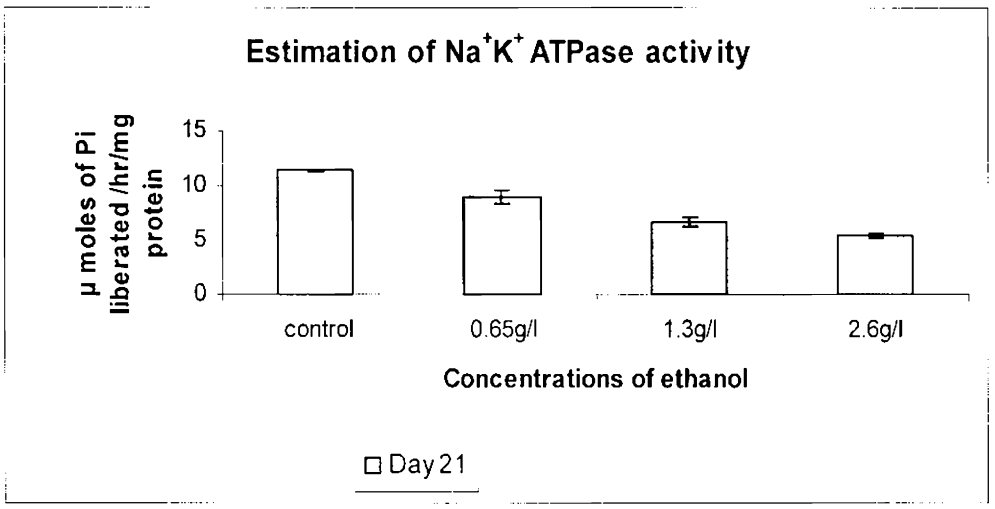
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	132.672	1	132.672	581.341	0.000
Between Concentrations	1095.286	3	365.095	1599.771	0.000
Days of exposure × Concentration	31.782	3	10.594	46.421	0.000
Error	9.129	40	0.228		
Total	1268.869	47			

df – degrees of freedom

Two Factor ANOVA table indicated that Total ATPase activity in gill tissue varied significantly ($P<0.001$) between days. Between concentrations also, a marked significant difference ($P<0.001$) was noted. When the interaction effects of both the days as well as concentrations were considered, there also significant difference ($P<0.001$) was seen.

Figure 2.3.4 Levels of Na^+/K^+ ATPase activity in the gill of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Na^+/K^+ ATPase activity was found to be decreased in all the three sub lethal concentrations of ethanol (Figure 2.3.4) with respect to control in the gill tissues of *O. mossambicus* in both durations of exposure. To validate this ANOVA was carried out and the results obtained are depicted below (Table 2.3.4a).

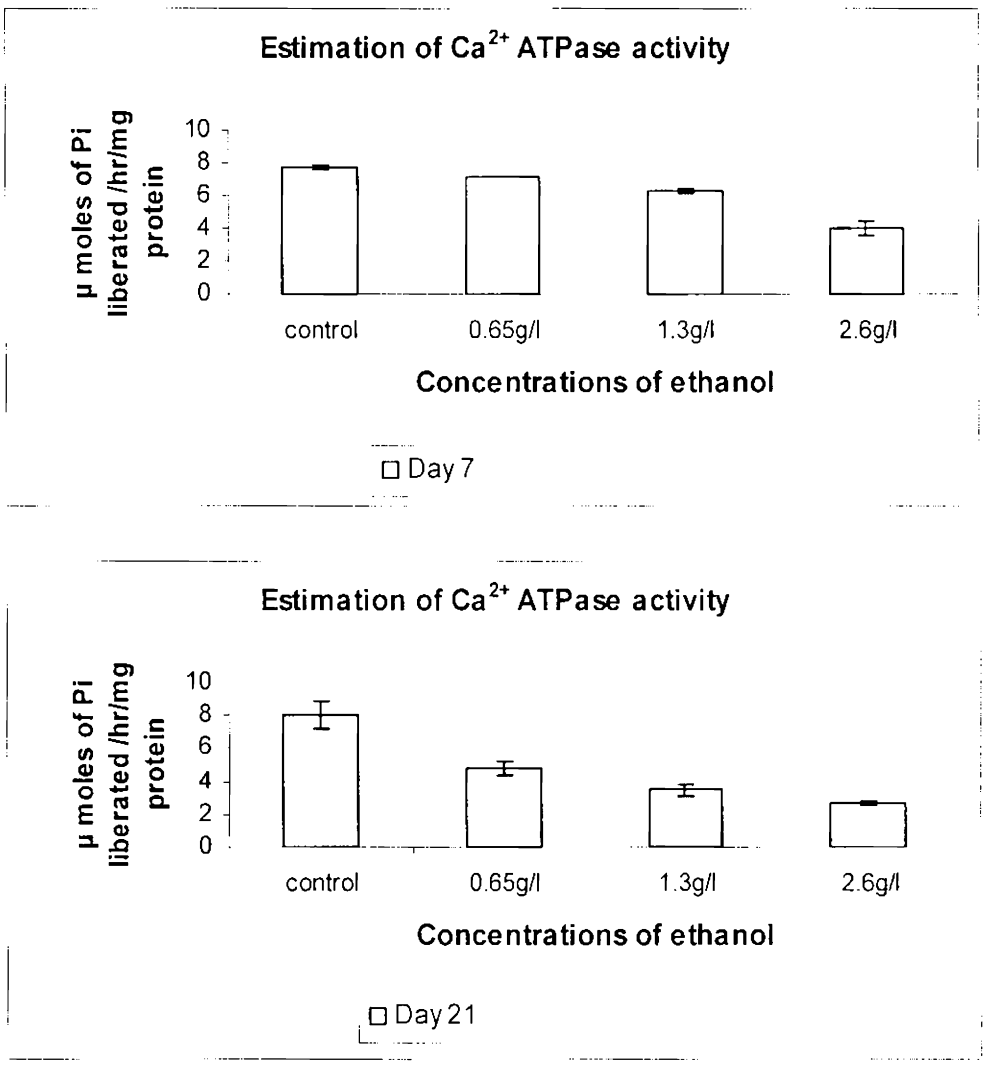
Table 2.3.4a ANOVA table for Na^+/ K^+ ATPase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	19.134	1	19.134	113.145	0.000
Between Concentrations	205.372	3	68.457	404.803	0.000
Days of exposure × Concentration	2.518	3	0.839	4.962	0.005
Error	6.765	40	0.169		
Total	233.789	47			

df – degrees of freedom

Statistical analysis carried out using Two Factor ANOVA table indicated that while considering the exposure between days, significant difference ($P<0.001$) was observed. While taking into consideration the effect between concentrations significant difference ($P<0.001$) was noted. Significant difference ($P<0.01$) was observed when the effect between both the days as well as concentrations were together taken into account.

Figure 2.3.5 Levels of Ca^{2+} ATPase activity in the gill of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Significant ($P<0.001$) (Figure 2.3.5) decrease in Ca^{2+} ATPase activity was observed in the gill tissues of *O. mossambicus* in all the three sub lethal dosage groups, with respect to control group on both 7 and 21 days. In order to substantiate the above statement ANOVA was taken into account and the results are mentioned below (Table 2.3.5a).

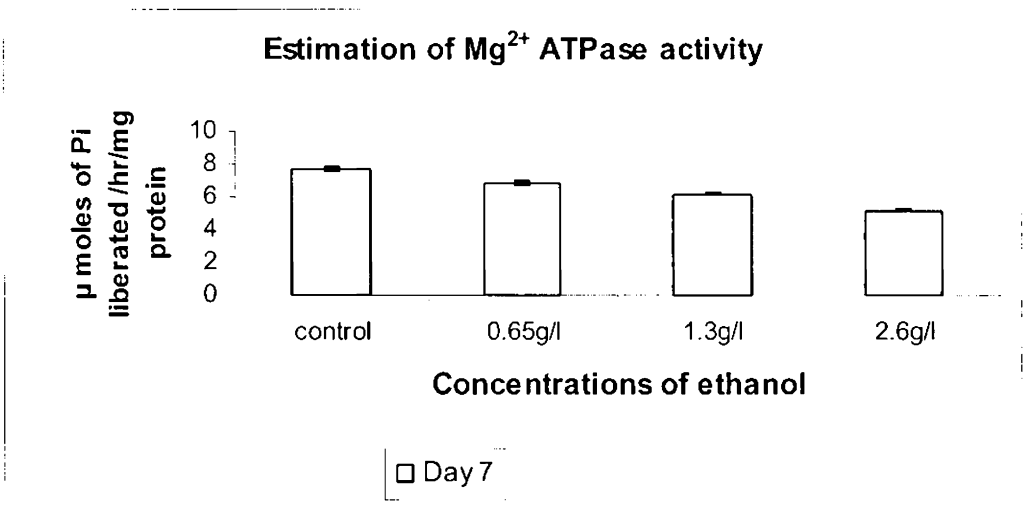
Table 2.3.5a ANOVA table for Ca^{2+} ATPase

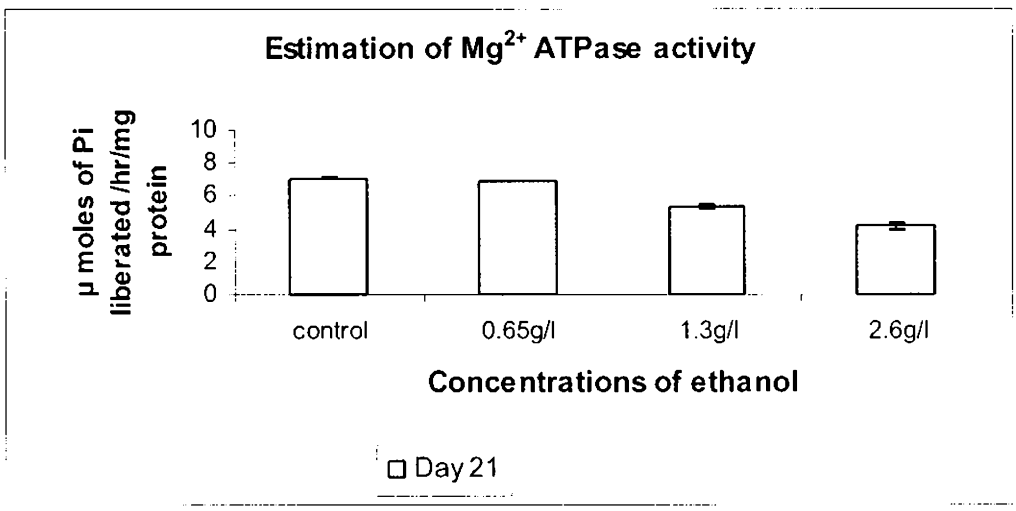
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	28.905	1	28.905	194.695	0.000
Between Concentrations	128.917	3	42.972	289.448	0.000
Days of exposure × Concentration	16.167	3	5.389	36.299	0.000
Error	5.939	40	0.148		
Total	179.928	47			

df – degrees of freedom

By using Two Factor ANOVA it was noted that Ca^{2+} ATPase level varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. On considering both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 2.3.6 Levels of Mg^{2+} ATPase activity in the gill of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Mg^{2+} ATPase activity was found to be significantly decreased ($P<0.001$) (Figure 2.3.6) when *O. mossambicus* was exposed for 7 and 21 days to various sub lethal ethanol concentrations with respect to control. This statement was supported by employing ANOVA and the result obtained is mentioned below (Table 2.3.6a).

Table 2.3.6a ANOVA table for Mg^{2+} ATPase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	4.123	1	4.123	273.612	0.000
Between Concentrations	52.334	3	17.445	1157.597	0.000
Days of exposure × Concentration	2.211	3	0.737	48.912	0.000
Error	0.603	40	0.015		
Total	59.271	47			

df – degrees of freedom

Studies done by using Two Factor ANOVA indicated that Mg^{2+} ATPase levels varied significantly between days ($P<0.001$). There was a significant difference ($P<0.001$) between concentrations also. While comparing both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was noted.

Table 2.3.7 Multiple Comparison Test

	Groups	Total ATPase	Na^+/K^+ ATPase	Ca^{2+} ATPase	Mg^{2+} ATPase
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = $P < 0.001$.

Subsequent pair wise comparison between various concentrations with respect to control using Dunnett's method revealed that Total ATPase, Na^+/K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase activity present in the gills of *O. mossambicus* exhibited significant difference ($P<0.001$).

2.4 Discussion

Osmoregulation in fish is influenced by external and endogenous factors (Evans, 1987). Among the external ones, the exposure to water-borne pollutants should be highlighted. Environmental toxicity can result in two types of structural changes. One is the direct toxic effect of the pollutant leading to tissue degeneration and necrosis, and the other is the development of compensatory mechanisms such as cellular hyperplasia to deal with the stressor (Bhagwant and Elahee, 2002; Hughes *et al.*, 1979). It has been observed that toxicity from pollutants may develop primarily from ATPase inhibition. ATPase is a type of lipid-protein, an important component in cell membrane. It catalyzes ATP to release energy, driving ion transport across membranes and maintaining a constant ion level in the animal body. Aquatic pollutants exert a biological effect on the ATPase system by partitioning in the enzyme complex, which may cause an allosteric change that result in decreased ATPase activity (Reddy *et al.*, 1992). The ATPases are considered to be involved in the maintenance of ionic balance in the gills of both fresh water and marine teleosts. Environmental organic pollutants usually affect the Na^+/K^+ ATPase by decreasing its activity (Haya *et al.*, 1983).

Damages in the membrane architecture may be the reason for the enzyme inhibition during the sub lethal treatment with ethanol. Another possible reason may be due to the non availability of substrates like ATP molecules which resulted in the

inhibition of ATPase. In the present study a significant decrease ($P<0.001$) (Figure 2.3.3 and Figure 2.3.3a) in the total ATPase activity of *O. mossambicus* was observed when exposed for 7 and 21 days to ethanol. Similar findings were cited by Jayantha Rao and John Sushma (2007) who too observed similar decrease in the total ATPase activity in the different tissues of albino mice when exposed to aluminium acetate. The observations made by Suhel *et al.* (2006) stated that there was a decrease in the total ATPase activity in the gills of freshwater fish *Channa punctata* (Bloch) when exposed to a diluted paper mill effluent for 15, 30 and 60 days which also supports the present study.

The membrane-bound transport enzyme Na^+/K^+ ATPase is an integral part of active transport mechanisms for cations across the cell membrane (Das and Mukherjee, 2000). Because ion-dependent ATPases are known to regulate the influx and efflux of ions across the membrane to maintain the physiological requirements of the cells, the inhibition of Na^+/K^+ ATPase in gills probably disturb Na^+ and K^+ pump, resulting in an uncontrollable entry of Na^+ into the cell along the concentration gradient, followed by the entry of water molecule along the osmotic gradient (Thaker *et al.*, 1996). The present results are in agreement with previous reports that ethanol brings about an inhibition in Na^+/K^+ ATPase activity. Although the exact mechanism underlying ethanol induced alterations in the activities of membrane-bound ATPase remains to be clarified, a combination of several possibilities with respect to the depressed ATPase activities may be considered. Biophysical studies indicate that ethanol alters membrane function by disintegrating the membrane and changing the mobility of membrane lipids and proteins. Acute exposure to ethanol has been demonstrated to exert a fluidizing effect on membranes by altering the fine structural arrangement of the lipid bilayer (Goldstein, 1983). This ethanol membrane interaction specifically affects some of the membrane-bound enzymes. Therefore, such morphological and/or ultra structural alterations may bring about changes in the conformational state of the ATPase molecule with a subsequent decrease in its activity. In addition, other factors such as impaired protein synthesis due to ethanol exposure may also contribute to this decreased enzyme activity. The findings put forth by Simkiss (1996) explain that inhibition of the gill Na^+/K^+ ATPase activity in fishes living in polluted rivers, slightly impaired the main biochemical systems without causing death during their experimental period of 30 days.

In the present study a significant decrease ($P<0.001$) (Figure 2.3.4 and Figure 2.3.4a) in the Na^+/K^+ ATPase activity of *O. mossambicus* was observed when exposed to 7 and 21 days. It was demonstrated that oxidative stress decreases the activity of Na^+/ K^+ ATPase. The decrease in the Na^+/K^+ ATPase activity in the gills of *O. mossambicus* during prolonged exposure may arise due to severe hypoxia which results in the reduction in ATPase activity. Similar decrease in Na^+/K^+ ATPase activity was supported by Ramon *et al.* (2002) who also observed decrease in Na^+/K^+ ATPase activity under acute ethanol intoxication in lungs and kidneys of rats. The findings of Hochachka and Lutz (2001) and Kim *et al.* (2008) also support the present data. It can also be inferred that the decrease observed in the Na^+/K^+ ATPase activity in the gills of *O. mossambicus* mainly refers to the changes in the membrane lipid content which in turn have been shown to influence the inhibition in Na^+/K^+ ATPase activity. Similar trend was observed in the gill tissues of freshwater fish *Channa punctata* (Bloch) when exposed to a diluted paper mill effluent for 15, 30 and 60 days (Suhel *et al.*, 2006). The decrease observed when *O. mossambicus* was subjected to different sub lethal concentrations of ethanol for 21 days was in turn supported by the findings of Bijoy *et al.* (2003) who had noticed a decrease in the Na^+/K^+ ATPase activity in the gill tissues in *Labeo rohita* when treated with synthetic detergent for 20 days. Inhibition of Na^+/K^+ ATPase activity may produce adverse effects in the organism (Yang *et al.*, 2002). Because inhibition of this enzyme occurs before gross osmoregulatory dysfunction, this would point out the use of Na^+/K^+ ATPase activity as an early indicator for pollutant induced damage to the ionic and osmoregulatory system (Stagg *et al.*, 1992a).

Ca^{2+} in the body plays an important role in keeping the membrane functioning normally. Ca^{2+} concentration in cells are maintained at a low level by ejecting Ca^{2+} out of the cell or absorbing Ca^{2+} into the endoplasmic reticulum and mitochondria across the membrane via Ca^{2+} ATPase. Several earlier studies have dealt with the effects of ethanol on Ca^{2+} ATPase activity. In the present study a significant decrease ($P<0.001$) (Figure 2.3.5 and Figure 2.3.5a) in the Ca^{2+} ATPase activity of *O. mossambicus* was observed when exposed to 7 and 21 days. Studies indicated that an acute injection of ethanol inhibits Ca^{2+} ATPase activity (Ross *et al.*, 1985). In particular, fish gills being sensitive to changes are referred to as important

indicators of waterborne toxicants thereby reducing oxygen consumption and disrupting its osmoregulatory function (Ghate and Mulherkar, 1979). The severity of gill damage depends on the concentration of toxicants and also on the time of exposure (Oliveira *et al.*, 1996). The reduction in the activities of ATPase was correlated to the altered ionic transport and decreased ATP break down which had impaired the metabolic and vital physiological activities (Arvindkumar preeti, 2008).

Mg^{2+} ATPase is used to regulate Mg^{2+} level, which is important for the physiological activity of membrane protein (Yang and Huang, 1996). Mg^{2+} ATPase enzyme is found in association with Na^{+}/K^{+} ATPase in fish. Mg^{2+} ATPase is responsible for transepithelial regulation of Mg^{2+} ions across the gill epithelium. It is also essential for the integrity of the cellular membrane and for the stabilization of branchial permeability (Reddy *et al.*, 1991). In the present study a significant decrease ($P<0.001$) (Figure 2.3.6 and Figure 2.3.6a) in the Mg^{2+} ATPase activity of *O. mossambicus* was observed when exposed for 7 and 21 days to various sub lethal concentrations of ethanol. The decrease observed in the present study refers to reduced ATP production which therefore results in disruption in cellular and ionic regulation. The findings of Racker *et al.* (1975) support the present data. Further ethanol suppresses the ATPase activities through activation of lipid peroxidation. The free radicals generated during the catalytic cycle of ethanol would have induced the peroxidation process in membrane lipids. The inhibition observed in the ATPase activities when *O. mossambicus* was exposed to ethanol for 21 days refers to the peroxidation occurring in the damaged tissues which brings about a change in the structure and inactivates a number of membrane bound enzymes and protein receptors which finally disrupts branchial membrane integrity. This statement was supported by Maridonneau *et al.* (1983); Sato and Yonei (1987). Integrity of membrane is very much essential to maintain cation homeostasis.

The present findings warrant future studies to explore ATPases as possible biomarkers of pollutant exposure in ecotoxicology. The major findings of the present experiment validate that ATPase activity can be taken as a meaningful index of cellular activity and forms a useful toxicological tool. Thus it may be concluded that fish gill can be used as a model system to study the effect of ethanol on ion transport mechanisms across cellular and epithelial membranes.

Chapter 3

EFFECT OF ETHANOL ON HAEMATOLOGICAL PARAMETERS OF *OREOCHROMIS MOSSAMBICUS* (PETERS)

Contents

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3.1A INTRODUCTION

Aquatic systems are highly vulnerable to pollution since they act as immediate sinks for the consequences of human activity always associated with the danger of accidental discharges or criminal negligence (Vutukuru, 2005). Cooney *et.al.* (2001) stated that aquatic lives are also affected by the clean up operations as well as indirectly through physical damage to the habitats in which plants and animals live. Human destructive influence on the aquatic environment is in the form of sub-lethal pollution, which results in chronic stress conditions that have negative effect on aquatic life (Mason, 1991). The use of volatile organic solvents has been one of the sources of environment pollution. Groundwater contamination by fuel spills has been magnified by the addition of oxygenates, such as ethanol and methyl-

tertiary-butyl ether (MTBE). These additives, although beneficial in reducing atmospheric pollution may, however, increase groundwater contamination. With the introduction of ethanol to gasoline and diesel fuels imminent and the move away from MTBE use in many states of the USA, Brazil etc, the environmental implications associated with ethanol additive fuels must be thoroughly investigated. An aqueous ethanol concentration above 10% is required for leaching to occur. For each gallon of ethanol being produced, typical ethanol plants produce 12 gallons of sewage-like effluent in the fermentation and distillation process which being dumped into streams, ultimately affects the aquatic system. The presence of ethanol in groundwater makes the scenario more complicated since ethanol can increase the plume length of the most toxic contaminants. With the expected increased use of ethanol as a gasoline oxygenate, this type of spill scenario could occur more frequently (Corseuil *et al.,* 2000). Haematological techniques are the most common methods employed to determine the sub-lethal effects of the pollutants (Larsson *et al.*, 1985). Blood serves as an important tool for studying the rapid changes in blood parameters of fishes since it is highly susceptible to environmental fluctuations (Blaxhall, 1972; Pandey and Pandey, 2001). Fish haematology had been an essential tool for the biologist as a frontline sensitive indicator of vital physiological and biological functions as well as status of nutrition, health, diseases, and stress in response to changing environmental conditions (Devi *et al.*, 2004). Fish blood, being a patho - physiological reflector of the whole body, is important in diagnosing the structural and functional status of the fish exposed to toxicants (Sampath *et al.*, 1998). In recent years, haematological variables have been used more extensively where clinical diagnosis of fish physiology was applied. This was used to determine the effects of external stressors and toxic substances brought about by the close association between the circulatory system and the external environment (Cech *et al.*, 1996). Taofik *et al.* (2008) stated the importance of haematological parameters because of their relationship with energy (blood glucose), respiration (RBC, PCV and Hb levels) and defense mechanism (WBC level). Keeping these in view, laboratory studies were conducted to ascertain the effect of sub lethal concentrations of ethanol on some blood parameters in *O. mossambicus*.

3.2B MATERIALS AND METHODS

Collection, maintenance, acclimatization of fish, determination of LC_{50} and bioassay method for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

3.2B.1 Preparation of blood sample for haematological studies

For conducting haematological studies, *O. mossambicus* of 10 ± 2 g were taken in three separate tubs which contained desired concentration of ethanol (0.65 g/l, 1.3 g/l and 2.6 g/l respectively) along with tap water. Six replicates were kept for each experiment. The experimental animals were exposed for 21 days with a periodical sampling at 7 days also. During the experimental period of 21 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. Any other factor likely to influence toxicity was nullified by maintaining a suitable control. On completion of fixed exposure period, blood was drawn from the common cardinal vein using 1ml sterile plastic insulin syringe (Smith *et al.*, 1952) (26mm gauge size) containing EDTA as an anticoagulant (1mg/ml) (Oser, 1976) and free flowing blood was collected for haematological studies. Various haematological parameters were determined by following the standard methodology of Praful Godkar and Darshan Godkar (2003).

3.2B.2 METHODS USED FOR THE HAEMATOLOGICAL STUDIES

The following parameters were studied under haematology.

a. Determination of RBC count

Principle

The blood specimen is diluted 1:200 with the RBC diluting fluid and cells are counted under high power (40 × objective) by using a counting chamber. The number of cells in undiluted blood are calculated and reported as the number of red cells per cubic mm (µl) of whole blood.

Requirements

Microscope, RBC pipette, Improved Neubauer counting chamber with cover glass, RBC diluting fluid consisting of sodium citrate 3 g, formalin 1ml, and distilled water 100 ml.

Procedure

RBC count was performed with a Neubauer counting chamber as described by Praful and Darshan (2003). The blood was collected in a vial containing ethylene diamine tetra acetic acid (EDTA) (1mg/ml of blood) as an anticoagulant. The blood was usually diluted 200 times with RBC diluting fluid for doing red cell count. The blood was drawn up to the 0.5 mark in the RBC pipette and immediately the diluting fluid was drawn up to the mark 101 (thus the dilution becomes 1:200). The solution was then mixed well by gentle shaking. It was then allowed to stand for 2-3 minutes. The counting chamber and the cover glass were cleaned thoroughly and then the counting chamber was placed on a flat surface with the cover slip over the ruled area. After mixing gently for a few seconds and then discarding the first few drops the counting chamber was then charged by holding the pipette at an angle of 40^0 with the tip touching the space between the cover slip and the counting chamber. By the capillary action the fluid enters the chamber. The fluid was then allowed to fill the chamber space avoiding air bubbles. It was then kept for 3 to 5 minutes for the settlement of RBCs. Afterwards the ruled area of the counting chamber was focused under low power for examining the distribution of red blood cells. It was then changed to high power and the numbers of RBCs were counted in the four corner squares and in the center small squares of the RBC area and the number of RBCs present per cubic millimeter of undiluted blood was calculated accordingly.

$$\text{Total red blood cells/cu mm } (\mu l) = \frac{\text{Number of red cells counted} \times \text{Dilution}}{\text{Area counted} \times \text{Depth of fluid}}$$

b. Determination of WBC Count

Principle

Glacial acetic acid lyses the red cells while gentian violet slightly stains the nuclei of the leucocytes. The blood specimen is diluted 1:20 in a WBC pipette with

the diluting fluid and the cells are counted under low power of the microscope by using a counting chamber. The number of cells in undiluted blood are reported per cu mm (µl) of whole blood.

Requirements

Microscope, WBC pipette, Improved Neubauer counting chamber with cover glass, WBC diluting fluid consisting of glacial acetic acid (2 ml), 1 or 2 drops of Gentian violet and distilled water (97 ml).

Procedure

WBC count was performed with a Neubauer's counting chamber as described by Praful and Darshan (2003). The blood was collected in a vial containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant (1mg/ml of blood). The blood was usually diluted 20 times with WBC diluting fluid for doing white cell count. The blood was drawn up to 0.5 mark in the WBC pipette and immediately the diluting fluid was drawn up to 11 mark (thus the dilution becomes 1:20). The solution was then mixed well by gentle shaking. It was then allowed to stand for 2 to 3 minutes. The counting chamber and the cover glass was cleaned thoroughly and then the counting chamber was placed on a flat surface with the cover slip over the ruled area. After mixing gently for a few seconds, and then discarding the first few drops the counting chamber was then charged by holding the pipette at an angle of 40^0 with the tip touching the space between the cover slip and the counting chamber. By the capillary action the fluid enters the chamber. The fluid was then allowed to fill the chamber space by avoiding air bubbles. It was then kept for 3 to 5 minutes for the settling of the cells. The total number of WBCs present in the four corner squares were counted using low power objective of the microscope. The number of WBCs present per cubic millimeter of undiluted blood was calculated accordingly.

$$\text{Number of white cells / cu mm (µl)} = \frac{\text{Number of white cells counted} \times \text{Dilution}}{\text{Area counted} \times \text{Depth of fluid}}$$

c. Determination of Packed Cell Volume (PCV) by Microhaematocrit method

Principle

Blood mixed with an anticoagulant was kept in a capillary tube. The tubes were then centrifuged at standard conditions, where the RBCs settle down at the bottom leaving a clear plasma column above. The ratio of the cells that were settled was expressed as the percentage of whole blood. This ratio is known as haematocrit or packed cell volume (PCV).

Requirements

Heparinized capillary tube, Blood containing anticoagulant.

Procedure

Microhaematocrit method employs small capillary tube of 8 cm length with a uniform bore size of 1mm diameter. Blood containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant (1mg/ml blood) was used. It was allowed to rise to about ½ to 3/4th of the even bored heparinized capillary tubes. The tubes were then sealed on the opposite end using sealing wax. The filled tubes were then placed in the radial grooves of the microhaematocrit centrifuge head with the sealed end being away from the centre. They were then centrifuged for 15 minutes at 12,000 ×g. PCV was then measured directly on a microhaematocrit reader associated with the centrifuge as Cell volume percent.

d. Estimation of Erythrocyte Sedimentation Rate (ESR) by Wintrobe's method

Principle

When blood containing anticoagulant was placed in a vertical tube undisturbed for a particular period of time, the erythrocytes tend to sediment towards the bottom giving two layers, an upper plasma layer and a lower red cell layer. This process is called erythrocyte sedimentation and the rate at which it occurs is called erythrocyte sedimentation rate or ESR. The length of the upper plasma column is expressed in millimeters per first one hour.

Requirements

Wintrobe tube, Wintrobe tube stand, Pasteur pipette or 2 ml syringe with needle, Timer or stop watch.

Procedure

Wintrobe's method employs Wintrobe's tube in which blood mixed with an anticoagulant (EDTA) in the ratio 1:1 was used. Blood was then filled up to zero mark, and it was then placed in a wintrobe stand in an exactly vertical position for about an hour. After 1 hour the height of the upper clear plasma column was measured and was reported in mm/hr.

e. Estimation of Fine packed red cells (FPRC)

Principle

Blood containing anticoagulant was placed in a Wintrobe tube and centrifuged. This in turn results in the settlement of erythrocytes. The scale on the right side indicates the packed red cells reading.

Requirements

Wintrobe tube, Wintrobe tube stand, Pasteur pipette or 2 ml syringe with needle, Timer or stop watch

Procedure

After reading the sedimentation rate the wintrobe tube was then centrifuged at 3000 rpm for 30 minutes, which in turn resulted in the settlement of erythrocytes. The scale on the right side indicates the packed red cells reading. Each line or mark on the tube represents the packed red cells in millimeter.

f. Determination of Haemoglobin (Hb) by Cyanmethaemoglobin method

Principle

The ferrous ions (Fe^{2+}) of haemoglobin are oxidized to ferric state (Fe^{3+}) by potassium ferricyanide to form methaemoglobin. The methaemoglobin then reacts with cyanide ions from potassium cyanide to form cyanmethaemoglobin which can then be measured colorimetrically.

Requirements

Methaemoglobin standard (60 mg%), Anticoagulated venous blood, Drabkin's reagent (This solution contains sodium bicarbonate 1 g, Potassium ferricyanide 200 mg, potassium cyanide 50 mg which was made up to 1 litre with distilled water).

Procedure

To 0.02 ml of blood, 5 ml of Drabkin's diluent solution was added. It was then mixed well and was allowed to stand for 5 to 10 minutes for the formation of cyanmethaemoglobin. Absorbance was then measured at 540 nm against a reagent blank which consisted of 5 ml of Drabkin's diluent solution. By using commercially available Cyanmethaemoglobin standard, a standard calibration curve was prepared from which the values of haemoglobin can be read directly as g/dl.

g. Determination of Erythrocyte Indices

Erythrocyte indices like MCV, MCH, MCHC were done as described by Dacie and Lewis,1991

1. Determination of Mean Corpuscular volume (MCV)

MCV or mean corpuscular volume is the average volume of red cells. It is calculated as follows

$$MCV = \frac{PCV \text{ in } \% \times 10}{RBC \text{ count in millions}}$$

2. Determination of Mean corpuscular haemoglobin (MCH)

Mean corpuscular haemoglobin or MCH is the average amount of haemoglobin in a red cell. It is calculated as follows

$$MCH = \frac{Hb \text{ in } \% \times 10}{RBC \text{ count in millions}}$$

3. Determination of Mean corpuscular haemoglobin concentration (MCHC)

Mean corpuscular haemoglobin concentration or MCHC is the average haemoglobin concentration in percentage per unit volume of packed red cells. It is the ratio of MCH to PCV or Hb to PCV expressed in percentage.

$$MCHC = \frac{MCH \times 100}{MCV \text{ or } Hb \times 100}$$

3.3C RESULTS

Effect of 0.65g/l, 1.3g/l, 2.6g/l concentrations of ethanol exposed for 21 days with a periodical sampling at 7 days also on the haemoglobin, red blood cell count, white blood cell count, packed cell volume, fine packed red cells, erythrocyte sedimentation rate, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration levels in the blood of *O. mossambicus* are given in tables 3.1 to 3.12 and in figures 3.3 to 3.11. Results were statistically analyzed by Two Way – ANOVA (Analysis of Variance) followed by Dunnett's method.

Table 3.1 Effect of exposure to different concentrations of ethanol for 7 days on haematological parameters of *O. mossambicus*.

Parameters	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Haemoglobin (g/dl)	3.20± 0.0894	2.73± 0.0516	3.37± 0.5164	5.50± 0.0894
RBC Count (10^6/Cu.mm)	0.67± 0.0274	0.53± 0.0164	0.80± 0.0055	1.02± 0.0876
WBC Count (10^3/Cu.mm)	7.88± 0.0219	7.68± 0.0274	7.69± 0.1032	7.85± 0.0745
ESR (mm/hr)	25.00± 0.8944	42.50± 1.6432	37.50± 0.5477	37.67± 0.5164
FPRC (mm)	9.00± 0.8944	6.50± 0.5477	13.20± 0.2191	14.40± 0.4382
PCV (%)	17.13± 0.8539	9.80± 0.2309	14.38± 0.6131	14.79± 0.4263
MCV (Cubic Microns)	5.48± 0.0316	5.23± 0.0411	5.19± 0.0443	4.95± 0.0574
MCH (Pico grams)	48.10± 0.3286	51.35± 0.6025	48.40± 0.6573	49.67± 0.5164
MCHC (%)	20.42± 1.1458	27.61± 0.2430	23.19± 2.8275	37.35± 1.8569

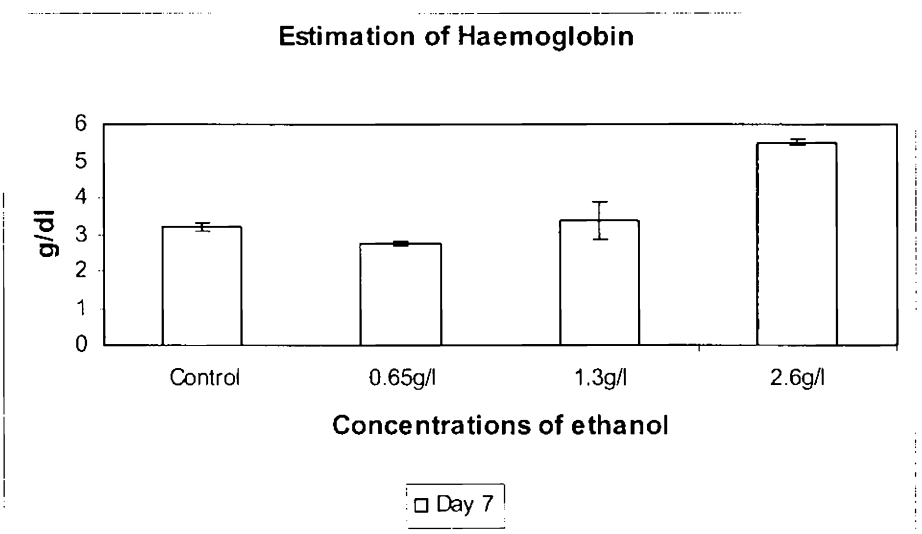
Average of six values in each groups ± SD of six observations.

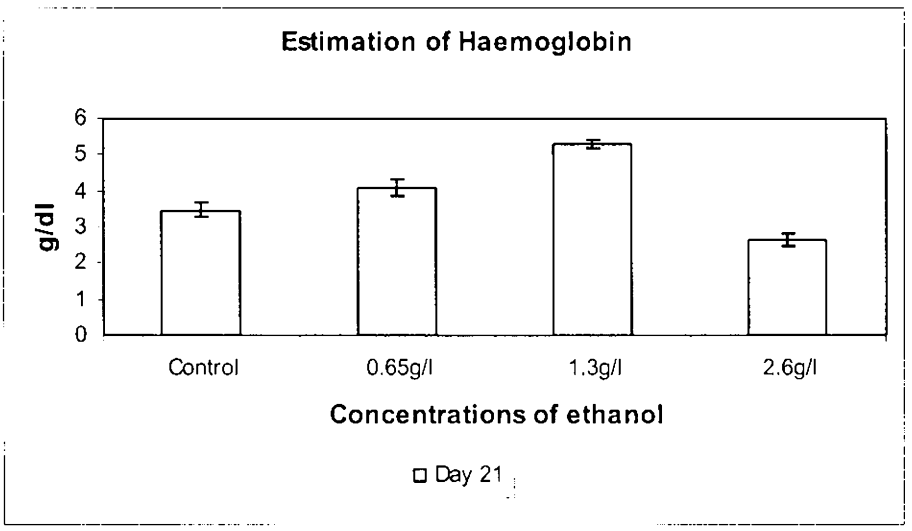
Table 3.2 Effect of exposure to different concentrations of ethanol for 21 days on haematological parameters of *O. mossambicus*.

Parameters	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Haemoglobin (g/dl)	3.47± 0.2066	4.10± 0.2191	5.30± 0.1096	2.65± 0.1643
RBC Count (10^6/Cu.mm)	0.75± 0.0274	0.81± 0.0323	1.18± 0.0164	0.56± 0.0219
WBC Count (10^3/Cu.mm)	7.76± 0.0219	8.15± 0.0822	7.60± 0.1096	7.24± 0.0767
ESR (mm/hr)	35.00± 0.6325	40.50± 0.5477	36.70± 0.3286	41.85± 0.1643
FPRC (mm)	10.33± 0.5164	15.20± 0.3098	19.50± 0.5477	12.50± 0.5477
PCV (%)	20.01± 1.1301	8.54± 0.1797	9.69± 0.4997	11.58± 0.3496
MCV (Cubic Microns)	5.54± 0.0265	4.66± 0.0365	4.42± 0.0680	5.33± 0.0625
MCH (Pico grams)	49.75± 1.0247	49.73± 1.5145	45.87± 0.7230	48.75± 0.4930
MCHC (%)	17.82± 1.9018	47.64± 1.6736	44.56± 0.6738	23.19± 0.6582

Average of six values in each groups ± SD of six observations.

Figure 3.3 Levels of haemoglobin in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Significant differences ($P<0.05$) were observed in the haemoglobin level of *O. mossambicus* exposed to 7 and 21 days (Figure 3.3). Statistical approach employing ANOVA corroborates this and can be inferred from the table below (Table 3.3a).

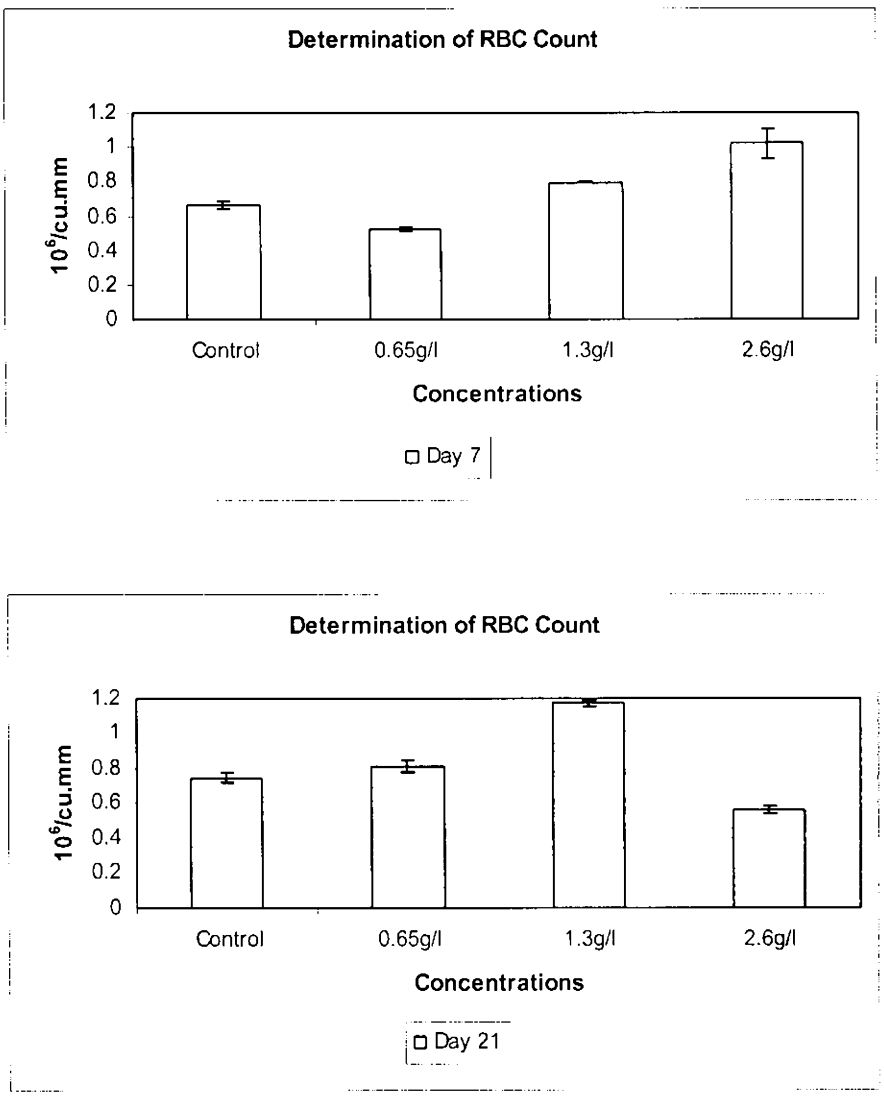
Table 3.3a ANOVA Table for haemoglobin

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.385	1	0.385	7.426	0.009
Between Concentrations	8.692	3	2.897	55.854	0.000
Days of Exposure × Concentration	41.012	3	13.671	263.533	0.000
Error	2.075	40	0.052		
Total	741.490	48			

df – degrees of freedom

Two Factor ANOVA table revealed that haemoglobin levels varied significantly between days ($P<0.01$). Between concentrations there was a significant difference ($P<0.001$). Also while taking into consideration the effects of both the days as well as concentrations (Interaction) significant difference observed was ($P<0.001$).

Figure 3.4 Levels of red blood cell count in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



O. mossambicus exhibited significant ($P<0.001$) alterations in the RBC Count value when subjected to various sub lethal concentrations of ethanol (Figure 3.4). Investigations using ANOVA substantiates the above statement which is shown below (Table 3.4a).

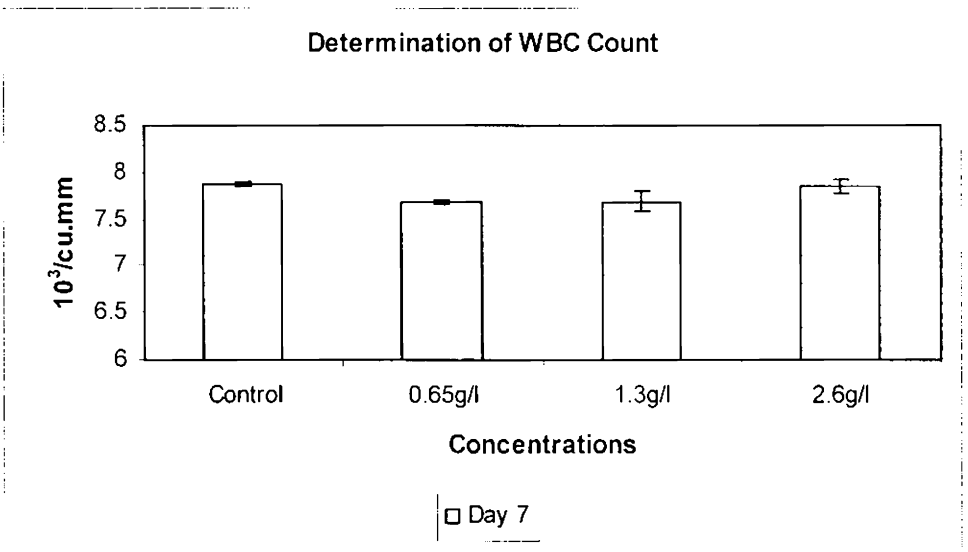
Table 3.4a ANOVA Table for RBC Count

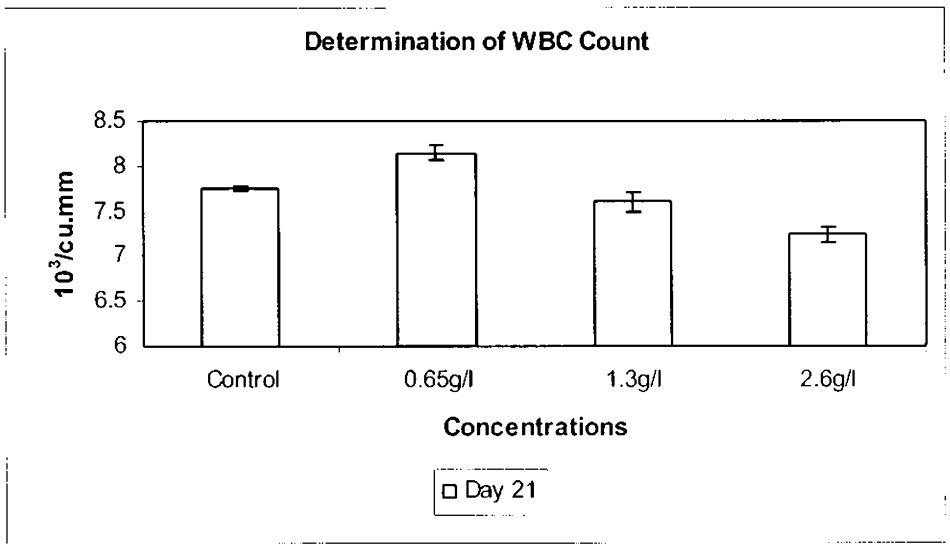
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.061	1	0.061	43.243	0.000
Between Concentrations	0.723	3	0.241	170.980	0.000
Days of Exposure × Concentration	1.270	3	0.423	300.492	0.000
Error	0.056	40	0.001		
Total	31.830	48			

df – degrees of freedom

Two Factor ANOVA table revealed that RBC Count varied significantly between days ($P<0.001$). Between concentrations there was a significant difference ($P<0.001$). Also while taking into consideration the effects of both the days as well as concentrations (Interaction) significant difference observed was ($P<0.001$).

Figure 3.5 Levels of white blood cell count in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Significant (P<0.001)alterations in WBC Count value was observed in the blood of *O. mossambicus* in all the three sub lethal groups with respect to control group on both 7 and 21 days of exposure(Figure 3.5). Anova was carried out to ascertain this statement and the table is depicted below (Table 3.5a).

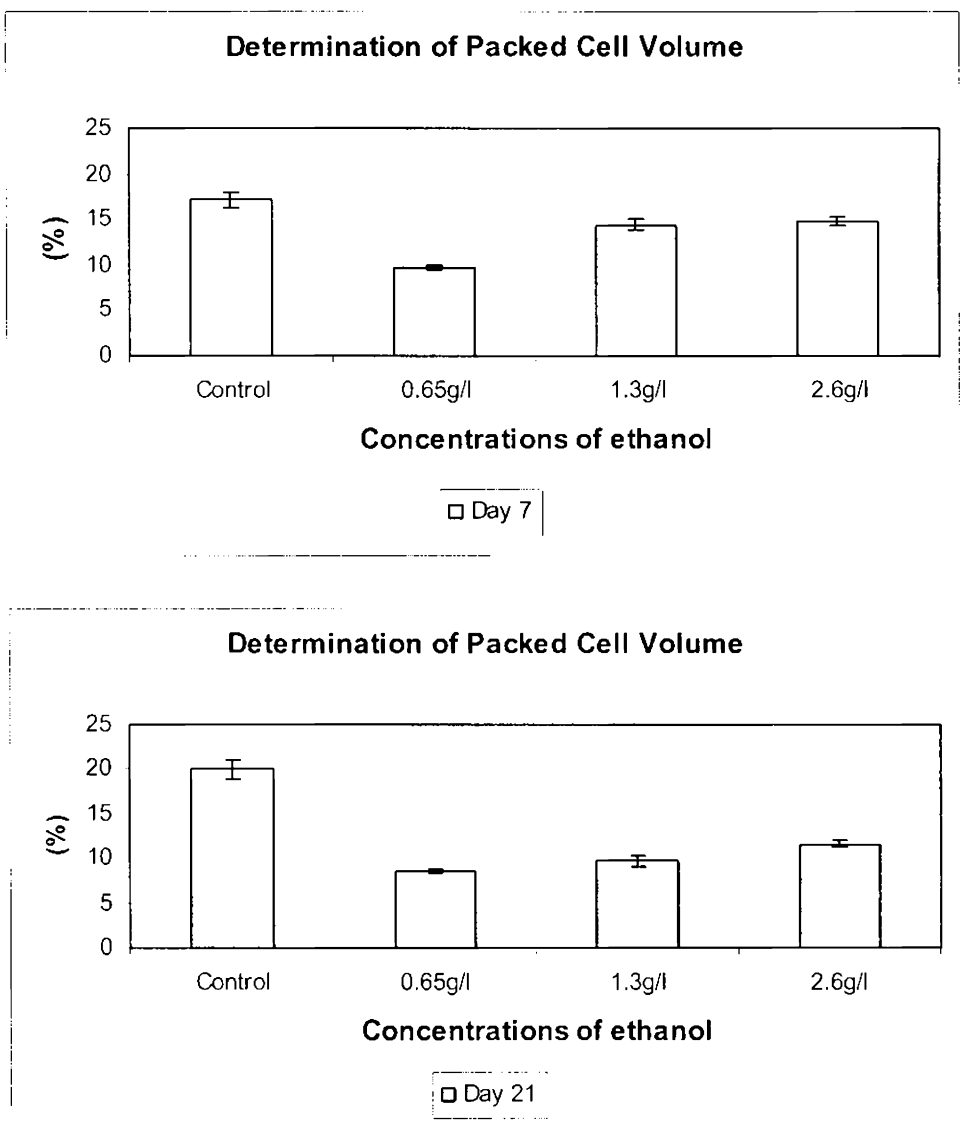
Table 3.5a ANOVA Table for WBC Count

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.093	1	0.093	17.570	0.000
Between Concentrations	0.983	3	0.328	62.007	0.000
Days of Exposure × Concentration	1.746	3	0.582	110.115	0.000
Error	0.211	40	0.005		
Total	3.033	47			

df – degrees of freedom

Two Factor ANOVA table revealed that in the case of WBC Count between days, significant difference (P<0.001) was observed. Between concentrations there was a significant difference (P<0.001). When taken into consideration the effects of both days as well as concentrations (interaction) significant difference observed was (P<0.001).

Figure 3.6 Levels of packed cell volume in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



A significant ($P<0.001$) decrease in packed cell volume (Figure 3.6) was noted in *O. mossambicus* exposed to various sub lethal concentrations of ethanol when compared to the control group. Statistical analysis conducted by using ANOVA substantiates this and the table is shown below (Table 3.6a).

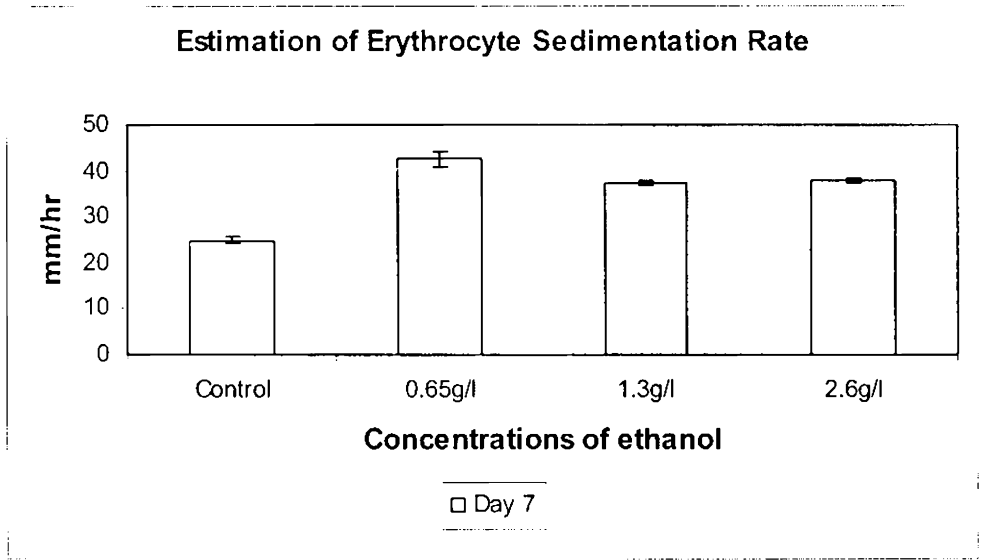
Table 3.6a ANOVA Table for PCV

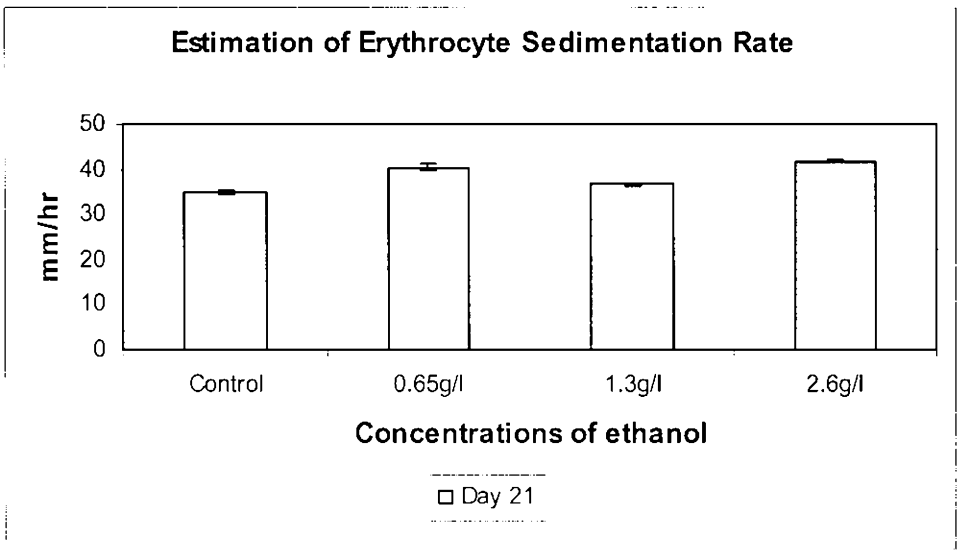
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	31.671	1	31.671	114.813	0.000
Between Concentrations	538.027	3	179.342	650.145	0.000
Days of Exposure × Concentration	89.288	3	29.763	107.894	0.000
Error	11.034	40	0.276		
Total	670.020	47			

df – degrees of freedom

Studies done by using Two Factor ANOVA indicated that Packed Cell Volume levels varied significantly between days ($P<0.001$). There was a significant difference ($P<0.001$) between concentrations. While comparing both the days as well as concentrations (Interaction), significant difference noted was ($P<0.001$).

Figure 3.7 Levels of erythrocyte sedimentation rate in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Erythrocyte sedimentation rate was found to be significantly increased ($P<0.001$) (Figure 3.7) when *O. mossambicus* was exposed for 7 and 21 days to various sub lethal ethanol concentrations with respect to control. Statistical analysis using ANOVA corroborated the above mentioned statement (Table 3.7a).

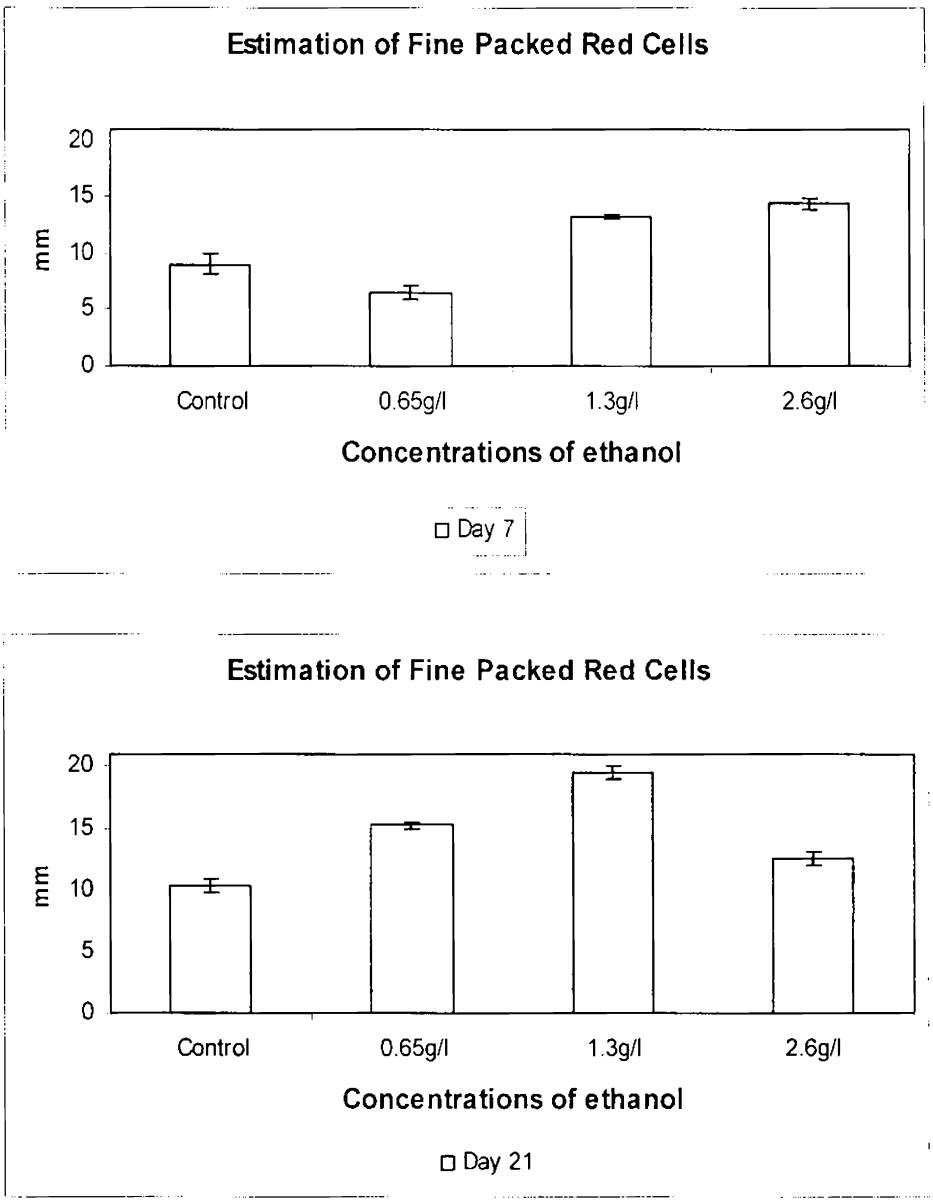
Table 3.7a ANOVA Table for ESR

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	97.185	1	97.185	158.616	0.000
Between Concentrations	922.036	3	307.345	501.617	0.000
Days of Exposure × Concentration	269.236	3	89.745	146.473	0.000
Error	24.508	40	0.613		
Total	67343.550	48			

df – degrees of freedom

By using Two Factor ANOVA it was noted that erythrocyte sedimentation rate level varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 3.8 Levels of fine packed red cells in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



From the graph (Figure 3.8) it can be concluded that fine packed red cell levels varied significantly ($P<0.001$) in *O. mossambicus* during immediate and prolonged exposure periods. Employing ANOVA justifies the above statement (Table 3.8a).

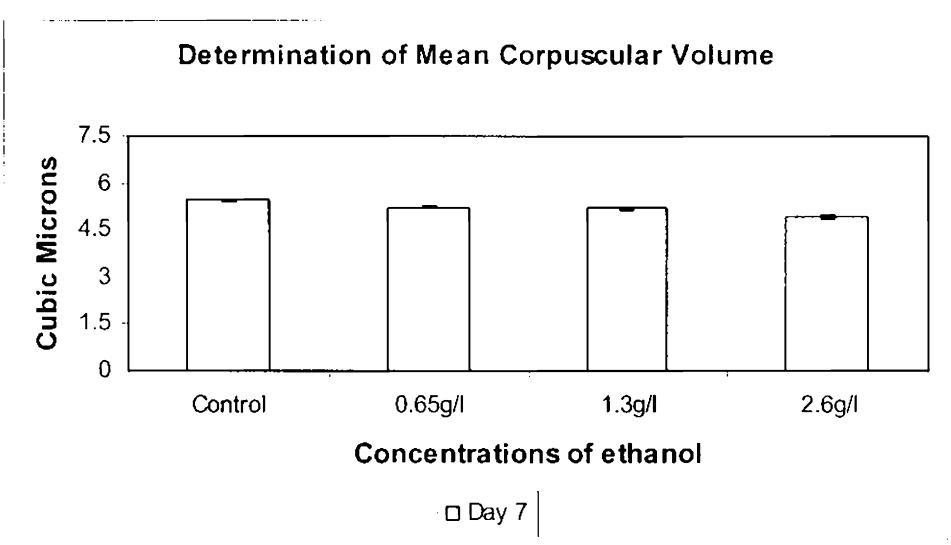
Table 3.8a ANOVA Table for FPRC

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	156.241	1	156.241	542.817	0.000
Between Concentrations	317.403	3	105.801	367.577	0.000
Days of Exposure × Concentration	206.062	3	68.687	238.636	0.000
Error	11.513	40	0.288		
Total	8286.520	48			

df – degrees of freedom

Statistical analysis done by using Two Factor ANOVA revealed that fine packed red cell levels varied significantly between days ($P<0.001$). It was noted that between concentrations significant difference was ($P<0.001$). It was concluded that by taking into consideration both the days as well as concentrations (Interaction) significant difference ($P<0.001$) was observed.

Figure 3.9 Levels of mean corpuscular volume in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



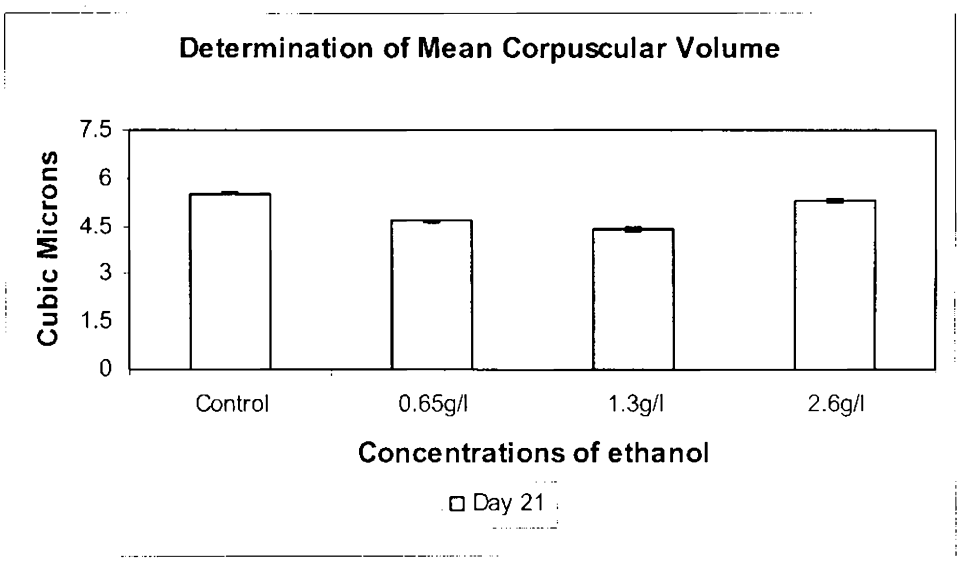


Figure 3.9 depicts significant decrease (P<0.001) in the mean corpuscular volume levels in *O. mossambicus* subjected to various sub lethal concentrations of ethanol during both the exposure periods. Analysis carried out by applying ANOVA supports the above statement (Table 3.9a).

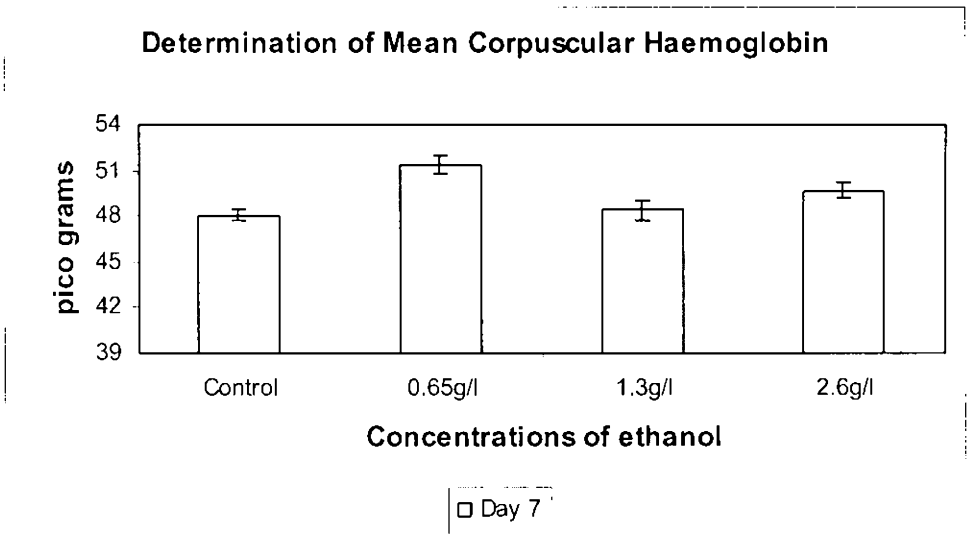
Table 3.9a ANOVA Table for MCV

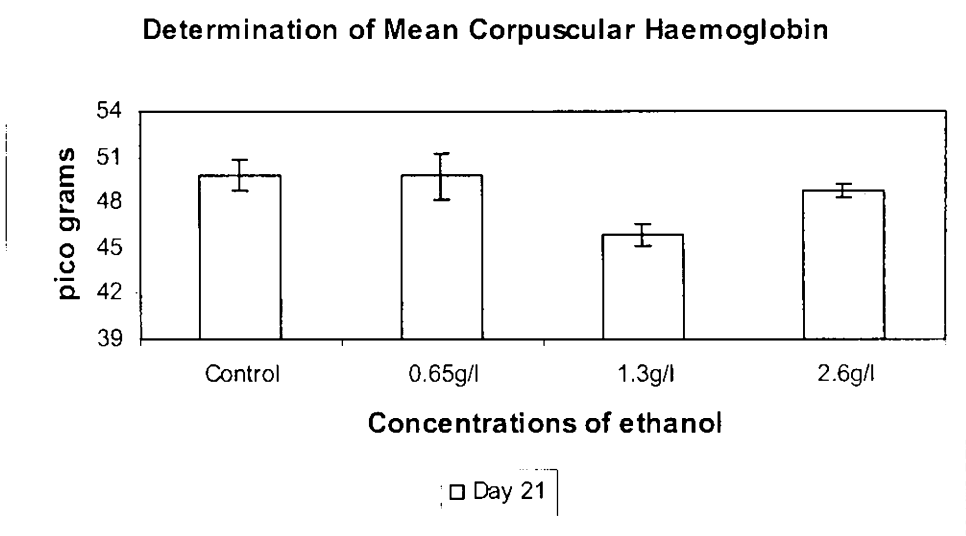
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.610	1	0.610	403.624	0.000
Between Concentrations	3.344	3	1.115	737.843	0.000
Days of Exposure × Concentration	2.552	3	0.851	563.145	0.000
Error	0.060	40	0.002		
Total	6.566	47			

df – degrees of freedom

Two Factor ANOVA table indicated that mean corpuscular volume levels varied significantly (P<0.001) between days. Between concentrations a marked significant difference (P<0.001) was noted. After considering the interaction effects of both the days as well as concentrations significant difference (P<0.001) was seen.

Figure 3.10 Levels of mean corpuscular haemoglobin in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





O. mossambicus exposed to various sub lethal concentrations of ethanol exhibited marked significant ($P<0.001$) (Figure 3.10) changes in the mean corpuscular haemoglobin value. The ANOVA table shown below (Table 3.10a) justifies this conclusion.

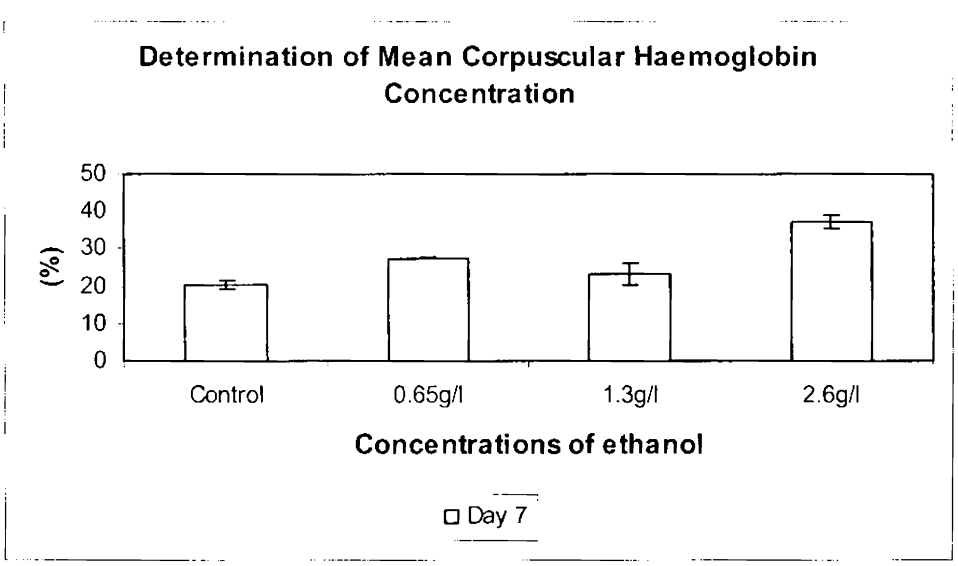
Table 3.10a ANOVA Table for MCH

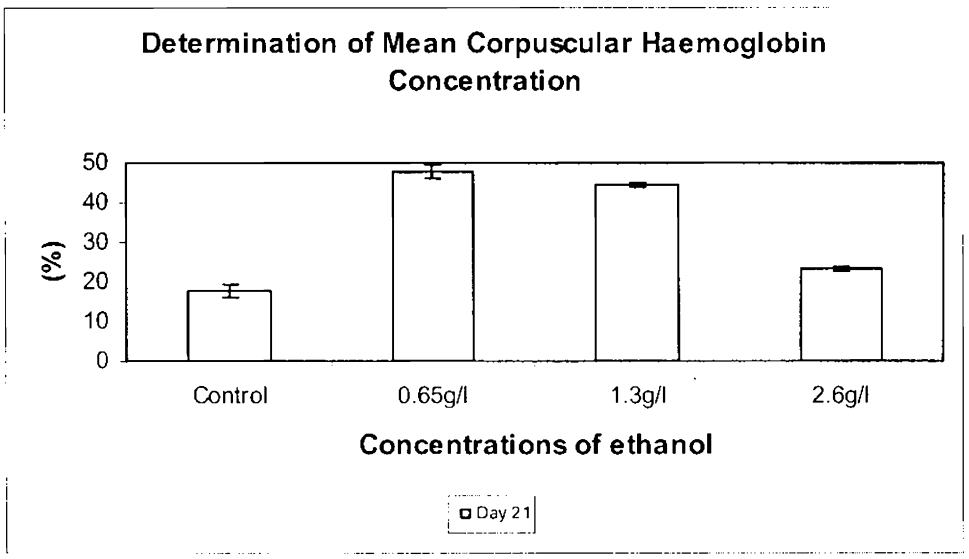
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	8.798	1	8.798	13.333	0.001
Between Concentrations	70.653	3	23.551	35.690	0.000
Days of Exposure × Concentration	29.066	3	9.689	14.682	0.000
Error	26.395	40	0.660		
Total	115152.728	48			

df – degrees of freedom

By using Two Factor ANOVA it was noted that mean corpuscular haemoglobin levels varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 3.11 Levels of mean corpuscular haemoglobin concentration in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Significant (P<0.001) changes were noted in the mean corpuscular haemoglobin concentration values (Figure 3.11) in *O. mossambicus* exposed to 7 and 21 days. Analysis using ANOVA authenticates this (Table 3.11a).

Table 3.11a ANOVA Table for MCHC

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	458.865	1	458.865	264.638	0.000
Between Concentrations	2288.665	3	762.888	439.975	0.000
Days of Exposure × Concentration	2748.013	3	916.004	528.280	0.000
Error	69.357	40	1.734		
Total	5564.900	47			

df – degrees of freedom

Studies done by using Two Factor ANOVA indicated that mean corpuscular haemoglobin concentration levels varied significantly between days (P<0.001). There was a significant difference (P<0.001) between concentrations. While comparing both the days as well as concentrations (Interaction), significant difference (P<0.001) was observed.

Table 3.12 Multiple Comparison Test

Subsequent comparisons by multiple comparison tests using Dunnett's is shown below.

Groups	Hb	RBC Count	WBC Count	ESR	FPRC	PCV	MCV	MCH	MCHC
Control Vs 0.65g/l	0.700[d]	0.049[c]	0.016[c]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.728[d]	0.000[a]

The values are significant at a = P < 0.001, b = P < 0.01 and c = P < 0.05 and not significant at d.

Hb - Haemoglobin, RBC - Red Blood Cell, WBC - White Blood Cell, ESR - Erythrocyte Sedimentation Rate, FPRC - Fine Packed Red Cells, PCV - Packed Cell Volume, MCV – Mean Corpuscular Volume, MCH - Mean Corpuscular Haemoglobin, MCHC - Mean Corpuscular Haemoglobin Concentration.

Subsequent comparisons by multiple comparison test using Dunnett's method is shown above (Table 3.12)

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. In the case of haemoglobin values no significant difference was observed at 0.65g/l with respect to control. In the case of RBC Count and WBC Count with respect to control significant difference ($P<0.05$) was observed at 0.65g/l. While considering ESR, FPRC, PCV, MCV and MCHC values significant difference ($P<0.001$) was observed in all the three concentrations with respect to control. Whereas in the case of MCH values with respect to control at 2.6 g/l no significant difference was observed.

3.4D DISCUSSION

Studies have shown that when the water quality is affected by toxicants, the physiological changes will be reflected in the values of one or more of the haematological parameters (Van Vuren, 1986). In recent years haematological

variables have been used more often to determine the sub lethal concentrations of pollutants (Wedemeyer and Yasutake, 1977). Ingestion of large amounts of ethanol results in a variety of toxicological consequences of which reduced oxygen supply is a marked respiratory effect. Erythrocytes are the most abundant cells in fish blood. They contain haemoglobin which in turn helps in carrying oxygen from gills to the different body parts (Johal and Grewal, 2004). In the present study a decrease in haemoglobin was observed at 0.65 g/l (Figure 3.3) when exposed to 7 days which is due to an increase in the release of immature cells from the haemopoictic tissues where as on prolonged exposure a decrease was observed at 2.6 g/l (Figure 3.3) which is an indication that anaemic condition occurred in fish during the exposure. This is due to a reduction in the absorption of iron from the gut. Iron is essential for the synthesis of haemoglobin. Anaemic conditions could also have resulted from the damage brought to the haemopoietic tissues such as the kidney and spleen. Prolonged exposure to ethanol inhibits haemoglobin synthesis and shortens the lifespan of erythrocytes. This present finding was supported by Alan (1995). Increase in haemoglobin level observed at 1.3 g/l and 2.6 g/l when *O. mossambicus* was subjected to ethanol exposure for 7 days is an indicator of fishes entering into a state of hypoxia. This mainly arose due to the damage occurred in the gills of the specimens. The increase in haemoglobin content may be to compensate the impaired respiration. This was supported by the findings of Chitra and Ramana (1986). Whereas a similar increase in the haemoglobin level exhibited by *O. mossambicus* on exposure to 21 days may have resulted due to excess production of erythrocytes to replace the oxidized or denatured haemoglobin. The findings of Sweety *et al.* (2008) supported the above statement. Similar pattern of reduction was observed in the case of RBC values (Figure 3.4) which in turn explain that both RBC and haemoglobin exhibit a direct physiological interrelationship. Sullivan and Herbert (1964) had explained the suppression of haematopoiesis brought about by ethanol. The same findings were supported by Balkaya *et al.* (2005) who also reported the effect of alcohol and passive smoking on some haematological variable of Swiss albino mice. The increase in the RBC Count (Figure 3.4) observed during both immediate as well as prolonged exposure to ethanol may be a reaction to the hypoxic conditions caused by epithelial lifting of the gill lamellae. This is supported

by Wepener (1990). Decrease in the RBC count value as well as increase in the haemoglobin level observed when *O. mossambicus* was subjected to immediate exposure indicates haemodilution of the blood which mainly arises due to damage brought to the organs of the fish, *O. mossambicus*. The changes occurring in the haematological parameters such as haemoglobin, RBC count, mean corpuscular volume can be interpreted as a compensatory response to improve the oxygen carrying capacity and maintain the gas transfer. This indicates a change in the water blood barrier for gas exchange occurring in the gill lamellae. This finding was supported by Velisek *et al.* (2008). Decreased value of RBC count observed when *O. mossambicus* was subjected to varying sub lethal concentrations of ethanol for 21 days suggest an anaemic condition occurring in the ethanol treated *O. mossambicus*. This may be attributed to cytotoxic effect and suppression of erythropoiesis caused by ethanol. Observations cited by Taofik *et al.*, (2008) supported this statement. A decrease in the WBC count values (Fig 3.5) was noted when ethanol subjected to 7 days of exposure. This may be due to the result of increased secretion of corticosteroid hormones. The secretion of these hormones is a non specific response to any environmental stressor and is a fundamental mechanism in the increased susceptibility of fish to disease when exposed to a pollutant. The observation in this study is similar to the findings of Ellis (1981), Murat Yalcin and Artay Yagci (2005). The increase in the WBC Count observed at 0.65 g/l (Figure 3.5) on prolonged exposure to ethanol is due to the stimulation of the immune system to protect the organism against infections. This is supported by the findings of Dede *et al.*, (2002). Taofik *et al.* (2008) also observed a similar pattern in the cat fish and rat when exposed to increased crude oil concentration. A decrease in the WBC count value observed when *O. mossambicus* was exposed for 21 days suggests that the fishes were exposed to a high risk of infection. Observations cited by Ngodigha *et al.* (1999) supported this finding. Similarly simultaneous increase in the WBC count value indicates leucocytosis, an adaptation made to cope with the stressful conditions due to the presence of ethanol. Sweety *et al.* (2008) supported this statement. The PCV values are considered to be important in determining the effect of stresses on the health of an animal. Hence PCV is referred to as an indicator of oxygen carrying capacity of the blood (Larsson *et al.*, 1985). A significant decrease

(P<0.001) (Table 3.6a) in the PCV value was observed when the fish was subjected to immediate and prolonged exposure to ethanol. The decrease in the PCV value obtained during immediate exposure (Figure 3.6) could be attributed to the gill damage where as decrease in the PCV value obtained during prolonged exposure (Figure 3.6) may be due to impaired osmoregulation brought about by ethanol affecting the haemopoietic system and biochemical pathway of haem formation leading to microcytic anaemia. This data is supported by the observations of Ahmad *et al.* (1995). Dede *et al.* (2002) explained that long-term exposure to petroleum samples particularly gasoline induces anaemia through the reduction in PCV levels. Similarly a significant decrease (P<0.001) (Table 3.9a) in the MCV levels were observed when the fish were subjected to immediate and prolonged exposure to ethanol. The decrease in the MCV value obtained during immediate exposure (Figure 3.9) may be due to the release of immature red blood cells from haemopoietic tissues. Immature cells are released to compensate for the loss of blood cells and increase the oxygen supply to relieve the hypoxic conditions which were experienced where as on prolonged exposure, a decrease in MCV levels (Figure 3.9) indicates the shrinkage of red blood cells brought about by microcytic anaemia. Similar results were reported by Adeyemo (2005) who observed a reduction in the MCV values in *Clarias gariepinus* when exposed to Cassava mill effluent. Muhammed and Telat (2003) also observed a similar decrease in PCV and MCV values when exposed to mancozeb which in turn explained that mancozeb treatment interferes with the normal physiology of red blood cells. ESR rate increased on both immediate as well as on prolonged exposure (Figure 3.7) to ethanol. This significant increase (P<0.001) (Table 3.7a) in the ESR rate indicates a possible pathologic condition. This supports the findings of Singh *et al.* (2002) who also observed an increase in the ESR value on *Heteropneustes fossilis* when exposed to linear alkyl benzene sulphonate. A subsequent increase (P<0.001) (Table 3.8a) in the levels of FPRC was observed which is due to an increase in the number of both immature and mature RBCs. The slight increase and decrease observed in the MCH (Figure 3.10), MCHC (Figure 3.11) values when subjected to immediate and prolonged exposure to ethanol may be attributed to a condition commonly associated with a decrease in number and increase in size and haemoglobin content

of RBCs suggesting a hyperchromic anaemic state. This present finding is supported by the observations of Taofik *et al.* (2008).

The present study reveals that ethanol has profound effect on fish blood parameters. Since blood is the most important body fluid, various physiological changes occurring in the body due to the toxicant will be reflected in the blood. Hence even slight variations in the aquatic environment may be reflected in the fish blood thus making it a sensitive indicator of pollution. Therefore, haematological studies are of ecological and physiological interest helping us to understand the relationship of blood characteristics to the habitat and adaptability of the species to the environment. Thus, it can be concluded that ethanol has serious consequences on haematological parameters in *O. mossambicus*.

Chapter 4

EFFECT OF ETHANOL ON SERUM TRACE ELEMENTS OF *OREOCHROMIS MOSSAMBICUS* (PETERS): STUDIES BY ATOMIC ABSORPTION SPECTROPHOTOMETRY.

Contents

4.1A Introduction

4.2B Materials and Methods

4.2B.1 Preparation of serum samples

4.2B.2 Estimation of serum trace elements

4.3C Results

4.4D Discussion

4.1A INTRODUCTION

The maintenance of a healthy aquatic ecosystem is dependent on the physico-chemical characteristics of water as well as on the biological diversity of the ecosystem. Urbanization, industrialization and depleting natural resources aggravated environmental degradation making it a global problem, resulting in environmental pollution affecting the aquatic life. The problems of environmental pollution and its deleterious effects on aquatic biota including fish have received focused interest during the last few decades. Industrial pollution, besides being a hazard to the human population is also responsible for the adverse effects on aquatic life, including fish (Saxena *et al.*, 1982). In view of the growing demand for ethanol, identifying the resources and developing economical methods required for the extraction of ethanol is very essential. The by - products formed during extraction procedure include several nutrients and organic matter which are difficult to handle and cause environmental pollution (Joshi and Devarajan, 2008). Ethanol enters the

environment as emissions from its manufacturing units, when it is used as a solvent and chemical intermediate, and when released in fermentation and alcoholic beverage preparation. High concentrations of ethanol occurring in spills or leaks at ethanol plant sites bring about acute effects on a wide range of aquatic biota. The use of ethanol as a fuel additive has increased the groundwater contamination problems (Natalie *et al.,* 2007).

Trace elements are generally chemical elements present or required in minute quantities. These in turn serve as essential components of biological enzyme systems as well as of structural components of biologically active constituents (Morton, 1975). They are also present in metallothionein (Zn), ceruloplasmin (Cu), super oxide dismutases (Cu, Se, Zn), and glutathione peroxidase (Se) (Figen Deveci and Nevin Ilhan, 2003). Feridun *et al.* (2006) stated that trace elements such as copper, zinc and selenium are recognized as essential mediators required for the development and progression of ethanol induced diseases. They also play an important role in the etiopathogenesis of the diseases brought about by ethanol. Any changes observed in the physiological as well as environmental conditions bring about fluctuations in the composition of trace elements present in fish serum. Trace metal determinations were performed by atomic absorption spectrophotometry (AAS) since they are accurate, specific, reproducible and reliable.

4.2B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study was the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

4.2B.1 Preparation of serum samples for the trace element studies

Blood was drawn from the common cardinal vein using 1ml sterile plastic insulin syringe (Smith *et al.*, 1952) of 26 mm gauge size and serum was separated from blood cells by centrifugation at 3000 rpm for 30 minutes. The separated serum was then kept stored at -20°C until assayed.

4.2B.2 Estimation of serum trace elements using atomic absorption spectrophotometry

Principle

In flame atomic absorption spectrometry, a sample is aspirated into a flame and atomized. A light beam is directed through the flame, into a monochromator, and then onto a detector that measures the amount of light absorbed by the atomized element in the flame. Since each metal has its own characteristic absorption wavelength, a source lamp composed of that element is used. This makes the method relatively free from spectral or radiation interferences. The amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of element in the sample over a limited concentration range.

Standard Atomic Absorption Conditions for copper (Cu), Zinc (Zn) and Selenium (Se).

Trace Element	Wave length (λ) nm	Slit Band Width (nm)	Flame	Instrument Detection Limit (mg/l)	Sensitivity (μg/ml)	Linear Range (mg/l)	Lamp current (mA)
Cu	324.8	0.7	A-Ac	0.07	0.1	5.0	15
Zn	213.9	0.7	A-Ac	0.018	0.02	1.0	15
Se	196.0	2.0	A-Ac	0.6	0.5	200	16

Reagents

Milli Q water, Conc. HNO_3: Conc. $HClO_4$ mixture (5:1), Stock solution of copper, zinc and selenium were prepared by diluting concentrated solution of copper, zinc and selenium (stock standards) of 1000 mg/l (Merck). All glassware and plastic materials used were previously treated for a week in 2 N Conc. HNO_3. They were then rinsed with double-distilled water and finally with Milli Q water.

Procedure

Trace elements were estimated according to the method of APHA (2005). To 1 ml of the serum sample, 5 ml of concentrated nitric acid and perchloric acid in the ratio 5:1 was added. It was then covered with a watch glass. The sample was kept at 70^0C overnight for digestion using a microwave digestion system. The completely digested samples were cooled to room temperature. It was then made up to 10ml using Milli Q water into a clean 10 ml standard flask. Digested samples were then analyzed using Perkin Elmer model 3100 atomic absorption spectrophotometer equipped with a Deuterium background corrector, for the determination of trace elements namely copper, zinc and selenium. Pure standards were also analyzed simultaneously and the data obtained were calculated based on the standard curves obtained. Serum selenium, copper and zinc values were expressed in mg/l.

4.3C Results

Effect of 0.65g/l, 1.3g/l and 2.6g/l concentrations of ethanol on the serum trace elements of *O. mossambicus* exposed for 21 days with a periodical sampling at 7 days are given in tables 4.1 to 4.6 and in figures 4.3 to 4.5. The trace elements analyzed were Cu, Zn and Se. Results were statistically analyzed by Two way ANOVA (Analysis of Variance) followed by Dunnett's method.

Table 4.1 Effect of exposure to different concentrations of ethanol for 7 days on serum trace elements of *O. mossambicus*.

Trace elements Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Copper (mg/l)	0.423± 0.0250	0.773± 0.0222	0.578± 0.0171	0.295± 0.0208
Zinc (mg/l)	17.803± 0.1377	17.420± 0.0216	17.128± 0.0411	16.670± 0.0623
Selenium (mg/l)	27.325± 0.6397	22.025± 1.4863	17.940± 0.9590	15.495± 1.0489

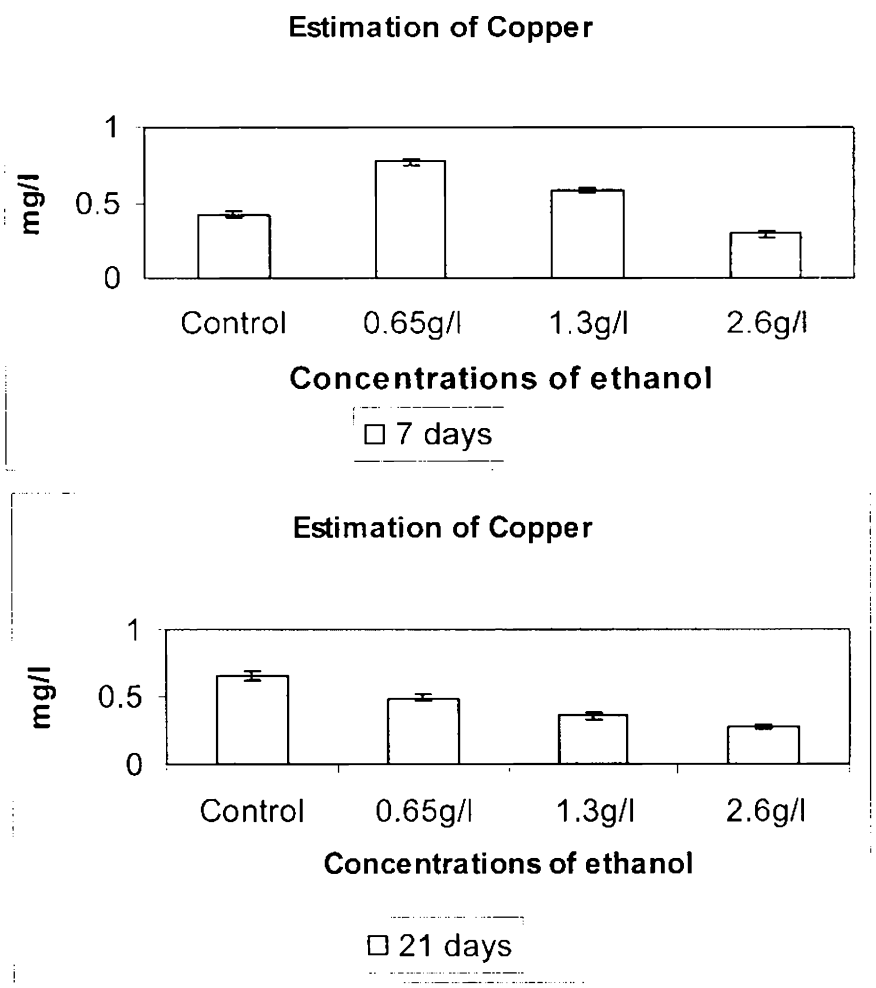
Average of six values in each group ± SD of six observations
Trace- elements (Cu, Zn and Se) were expressed as mg/l

Table 4.2 Effect of exposure to different concentrations of ethanol for 21 days on serum trace elements of *O. mossambicus*.

Trace elements Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Copper (mg/l)	0.663± 0.0350	0.490± 0.0245	0.358± 0.0287	0.273± 0.0222
Zinc (mg/l)	15.598± 0.0330	16.575± 0.0480	20.233± 0.8306	21.938± 1.3337
Selenium (mg/l)	31.500± 1.2910	40.075± 2.9748	51.675± 2.0614	61.550± 2.0825

Average of six values in each group ± SD of six observations
Trace- elements (Cu, Zn and Se) were expressed as mg/l

Figure 4.3 Levels of copper in the serum of O. *mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Highly significant (P<0.001) alterations were observed in different sub lethal concentrations of the treatment group when compared to control group of *O. mossambicus* during exposure periods of 7 and 21 days (Figure 4.3). Statistical approach using ANOVA substantiates the above statement (Table 4.3a).

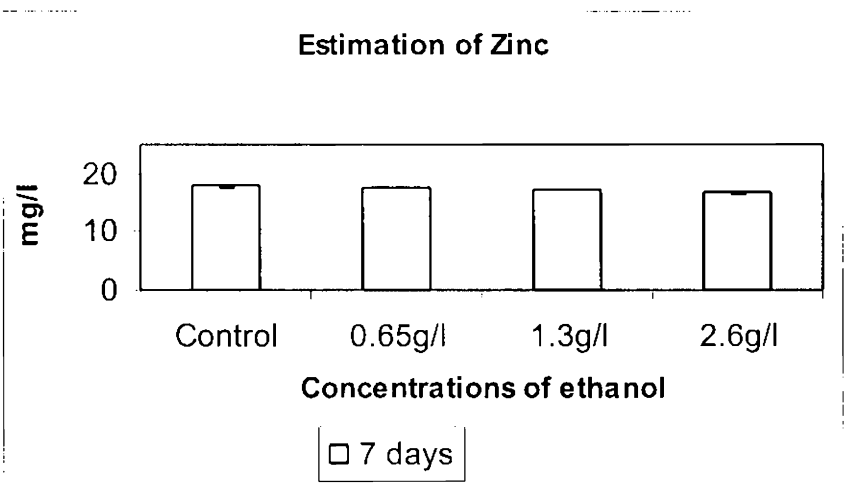
Table 4.3a ANOVA Table for Copper

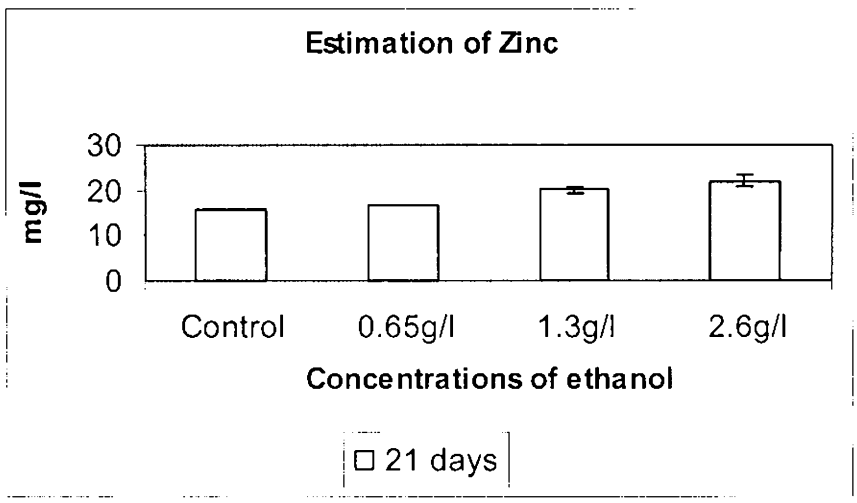
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.041	1	0.041	65.197	0.000
Between Concentrations	0.524	3	0.175	280.174	0.000
Days of Exposure × Concentration	0.332	3	0.111	177.666	0.000
Error	0.015	24	0.001		
Total	0.911	31			

df - degrees of freedom

Two Factor ANOVA table revealed that serum copper levels varied significantly between days (P<0.001). Between concentrations there was a significant difference (P<0.001). While taking into consideration the interaction effect of both the days as well as concentrations a significant difference (P<0.001) was observed.

Figure 4.4 Levels of zinc in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Significant differences (P<0.001) in serum zinc level (Figure 4.4) were observed in *O. mossambicus* exposed to all the three sub lethal concentrations of ethanol with respect to control group. To validate this ANOVA was carried out and the results are depicted below (Table 4.4a).

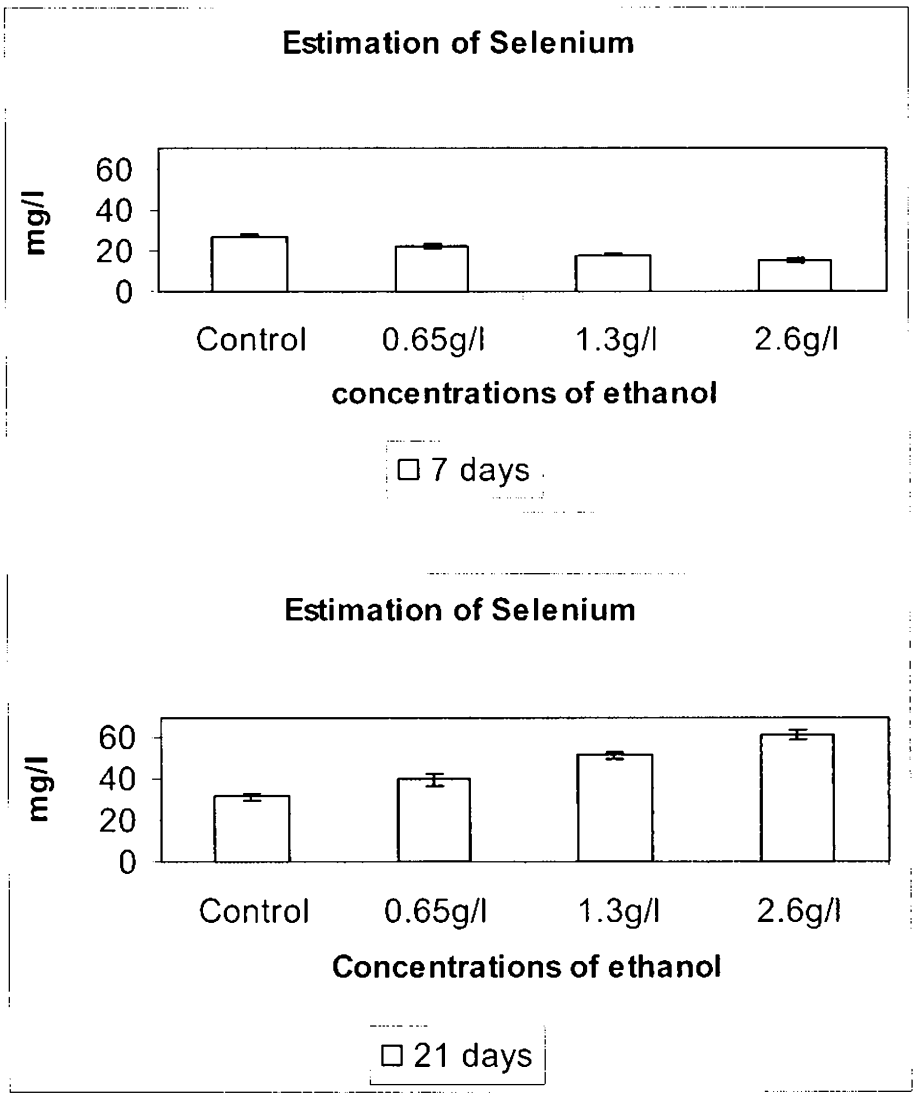
Table 4.4a ANOVA Table for Zinc

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	14.165	1	14.165	44.978	0.000
Between Concentrations	38.654	3	12.885	40.914	0.000
Days of Exposure × Concentration	71.763	3	23.921	75.959	0.000
Error	7.558	24	0.315		
Total	132.139	31			

df - degrees of freedom

Studies conducted on serum zinc levels exhibited a significant difference between days (P<0.001). Between concentrations there was a significant difference (P<0.001). Considering the interaction effect of both days as well as concentrations, a significant difference (P<0.001) was observed.

Figure 4.5 Levels of selenium in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



O. mossambicus treated with various ethanol concentrations exhibited a significant ($P<0.001$) alteration in serum selenium level when compared to the control groups on both periods of exposure. ANOVA was carried out to ascertain the statement and the table is shown below (Table 4.5a).

Table 4.5a ANOVA Table for Selenium

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	5203.530	1	5203.530	1753.519	0.000
Between Concentrations	397.076	3	132.359	44.603	0.000
Days of Exposure × Concentration	2001.163	3	667.054	224.788	0.000
Error	71.219	24	2.967		
Total	7672.988	31			

df - degrees of freedom

Two way ANOVA table indicated that serum selenium levels varied significantly between days (P<0.001). Between concentrations significant difference observed was (P<0.001). Also while taking into consideration the interaction effect of both the days as well as concentrations; significant difference observed was (P<0.001).

Table 4.6 Multiple Comparison Test

	Groups	Copper	Zinc	Selenium
Dunnett	Control Vs 0.65g/l	0.000[a]	0.591[b]	0.166[b]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001 and not significant at b.

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. In the case of serum copper values, significant difference (P<0.001) was observed in all the three concentrations with respect to control. While taking into consideration serum zinc and selenium values no significant difference was observed at 0.65 g/l with respect to control, whereas in the case of 1.3 g/l and 2.6 g/l significant difference (P<0.001) was observed.

4.4D Discussion

Alterations in the serum concentrations of trace elements have been extensively investigated. In the case of fishes, there is no report so far carried out on ethanol induced toxicity studies. Hence the present work was carried out to fill the gap. Selenium, zinc and copper are trace elements which play important role in biological systems as components of proteins, enzymes and antioxidants (Tomas *et al.*, 2001). Copper being an essential trace element and component of many enzymes, plays an important role in the biochemical process (Wojciech *et al.*, 2000). Donald (1999) explained copper as an important catalyst required for haemoglobin synthesis, involved in the absorption, storage, and metabolism of iron. Hasssan *et al.* (2006) have stated that copper is a component of superoxide dismutase (SOD), and it also requires zinc and manganese to function. Copper has been found in many proteins such as albumin, ceruloplasmin, metallothionein etc (Thiele, 2003). In the present study an increase in serum copper level at 0.65 g/l and 1.3 g/l followed by a decrease at 2.6 g/l (Figure 4.3) was observed on exposure to ethanol for 7 days with respect to control. The decrease in serum copper concentration of fish observed during 7 days of exposure at 2.6 g/l ethanol (Figure 4.3) may be due to the deficiency of copper brought about by the decreased intake of food. This present finding is supported by the observations of Morgan (1980) who observed a similar decrease in the serum copper levels in the hair of alcoholic patients. Similarly an increase in the serum copper levels was observed in fish exposed to 0.65 g/l and 1.3 g/l (Figure 4.3) ethanol which may be due to an increase in the copper binding component such as ceruloplasmin. This observation is supported by the findings of Linder and Hazegh (1996) who too observed an increase in the serum copper levels of cancer patients. On exposure to 21 days an overall decrease (Figure 4.3) was observed in the case of serum copper levels with respect to control. This indicated the deficiency of copper which in turn caused an increase in the synthesis of cholesterol leading to hypercholesterolemia. This present finding was supported by the observations of Strain *et al.* (2000) who observed a similar decrease in the serum copper levels of mice resulting in hypercholesterolemia condition. Similarly Mazur *et al.* (1992) suggested that the hypercholesterolemia associated with copper

deficiency was due to the result of selective increase in the HDL-1 sub fraction. Zinc, another trace element is regarded as an integral part of bio membranes which controls membrane integrity and stability. Carol (1999) stated the importance of zinc for growth, development and immune function. Zinc has also been involved in enzymatic functions like protein synthesis and carbohydrate metabolism. In the case of serum, zinc was bound predominantly to albumin (about 85%), and to a lesser extent to alpha 2-macroglobulin (about 16%) and then to amino acids (1% to 2%) (Foote and Delves, 1984). In the case of serum zinc level a decrease (Figure 4.4) was observed with respect to control on exposure to ethanol for 7 days. This is probably due to the disturbed immune system which indicates a diseased state. This is supported by Tomas *et al.* (2001) who observed a similar decrease in the serum zinc level in alcoholic patients with liver injury. Ioannis *et al.* (2007) have stated that a decrease in serum zinc level was observed in acute condition indicating the formation of fatty liver in alcoholic patients which shows that liver diseases affect the serum zinc levels. This too supported the present finding. In the case of exposure to ethanol for 21 days, an increase in the serum zinc level (Figure 4.4) was observed when being compared with control. This is due to the increase in levels of triglycerides, cholesterol and low density lipoprotein as well as decrease in the HDL levels which indicated enhanced cholesterol and triglyceride synthesis. This is in turn supported by He *et al.* (1994) who found a similar increase in serum zinc level in cardiac patients. Yurong Tang *et al.* (2003) also observed a similar increase in the serum zinc levels arising with hypertension as well as with coronary artery disease which also supported the present finding. Selenium being an essential trace element is the primary component of selenoproteins, which have roles in counteracting oxidative stress and regulating the redox status of other molecules. Selenium also influences the immune system. It has also been recognized as a structural component of glutathione peroxidase present in cytosol and mitochondria, protecting biomembranes against destruction which is one among the major antioxidant systems of the organism (Shivaprakash *et al.*, 2007). Serum selenium levels in fish subjected to 7 days of exposure to ethanol exhibited significant decrease ($P<0.001$) (Table 4.5a). This probably resulted from an increased protein damage brought about by an increased oxidative stress. This in turn resulted in the liver function

impairment leading to the development and progression of various diseases suggesting a state of cirrhosis. This observation is supported by the findings of Emilio *et al.* (2008) who found a similar decrease in the serum selenium level in alcoholics. On 21 days of exposure to ethanol a significant increase ($P<0.001$) (Table 4.5a) was observed in serum selenium which indicated increased glutathione peroxidase activities which in turn helped to attenuate the oxidative tissue damage. This was in agreement with the findings of Zuo *et al.* (2006) who too observed an increase in serum selenium level in leukemia patients which in turn explained an inverse relationship between serum selenium and disease activity in leukemia.

The present study revealed that ethanol had profound effect on serum trace elements especially on copper, zinc and selenium in *O. mossambicus* and found that they were significant at 0.1% level.

Chapter 5

5.1 EFFECT OF ETHANOL ON SELECTED SERUMPARAMETERS OF *OREOCHROMIS MOSSAMBICUS* (PETERS)

Contents

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d. Estimation of serum aspartate transaminase
e. Estimation of serum alkaline phosphatase
f. Estimation serum total protein

5.1C Results
5.1D Discussion

5.1A Introduction

Environmental pollution and its effects on the health of aquatic ecosystems have been studied intensely in the last years. With increasing industrial development, safe disposal of industrial wastewater seems to be one of the major ecological challenges. Polluted water bodies exert extensive stress upon aquatic animals (Barton and Iwama, 1991) Therefore, environmental degradation has now become a global problem and maintaining ecosystem health is a serious issue confronted by the environmentalists. Most of the industrial wastes discharged into the aquatic environment contain organic and inorganic pollutants in dissolved, suspended and insoluble forms (Dix, 1981). Disposal of industrial effluents into fresh water bodies deteriorates water quality, which is necessary to sustain aquatic life, primary productivity and food chain (Rao *et al.*, 2001). The water bodies are polluted mainly due to the discharge of wastes from various industries like distilleries, tanneries, fertilizers, pulp and paper, pharmaceuticals etc (Mangala *et al.*, 2002). The liquid wastes discharged by these industries contain large amounts of various chemicals

like phosphates, carbonates, alcohols, metals and organic compounds which seem to be lethal to aquatic flora and fauna (Kudesia, 1980). The ability of the pollutant to disrupt the biological balance as well as to cause deleterious effect on the aquatic ecosystem is dependent on the concentrations of the contaminant and the physico-chemical characteristics of the waste water effluent. The impact of contaminants on aquatic ecosystem is evaluated by measuring biochemical parameters in fish that respond specifically to the degree and type of contamination (Petrivalsky *et al.*, 1997). The impairment of water bodies by unrestricted disposal of industrial effluent makes it imperative to adopt short term tests for adequate and prompt monitoring of the aquatic ecosystem (Chukwu and Ogunmodede, 2005). The responses of aquatic organisms to pollution are demonstrated by changes in the expression of several key enzymes, especially those of biotransformation systems. Several specific enzymes have been proposed for monitoring water pollution (Agradi *et al.*, 2000). Serum enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST) are considered to be important serum markers to investigate the health of animal species of concern. Therefore, it is emphasized that measurement of serum biochemical parameters can be useful as a diagnostic tool in fish toxicology to identify their general health status and target organs affected by toxicants (Mustafa *et al.*, 2008). According to Musa and Omoregie (1999) fishes are intimately associated with an aqueous environment, any physical and chemical changes in the environment are rapidly reflected as measurable physiological changes in fish. The measurement of fish cellular enzymes is an indicator of health condition and has been used as diagnostic tool in monitoring programs of aquatic pollution (Oluah, 1999; Bernet *et al.*, 2001; Teles *et al.*, 2003; Begum, 2004). Toxicological studies have shown that the concentration of pollutants can change the enzyme activities and often directly induce cell damage in specific organs (Yang and Chen, 2003). Blood chemistry has long been a helpful diagnostic tool in pathological, toxicological and general clinical tests. Until recently, there were few attempts to introduce these techniques in aquatic toxicology studies (Raccicot *et al.*, 1975; Mehrle and Mayer, 1980; Casillas and Ames, 1986; Casillas *et al.*, 1983). As with mammals, it was proved that after functional damage to the tissues and organs of fish, some specific cellular enzymes would leak into the blood where they could

be detected (Bouck, 1966; Bouck *et al.*, 1975). Aalanine aminotransferase (ALAT, formerly SGPT), aspartate aminotransferase (ASAT, formerly SGOT), lactate dehydrogenase (LDH), creatine kinase (CK) and alkaline phosphatase (ALP) were selected as relevant enzymes for evaluating liver intoxication (Krajnovic, 1992). Haematology and serum enzyme evaluation of blood provide valuable information concerning the physiological responses of fish to changes in the external environment (temperature, pH, salinity and photoperiod) and thus serve as indicators of sub lethal environmental stress (Rajeev Kapila *et al.*, 2007). The aim of the present study is to investigate the changes in the various serum biochemical parameters, i.e. enzymes (ALT, AST, ALP and CK) and total protein in fishes exposed to various sub lethal concentrations of ethanol.

5.1B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

5.1B.1 Preparation of serum samples for experimental studies

Blood was drawn from the common cardinal vein using 1ml sterile plastic insulin syringe (Smith *et al.*, 1952) of 26 mm gauge size. The collected blood was then kept at room temperature for 30 minutes to obtain the serum. The serum thus obtained was then subjected to centrifugation at 3000 rpm for 30 minutes. The separated serum was then stored at -20°C until assayed.

5.1B.2 Methods used for serum experiments

The following parameters were studied under serum experiments

a. Estimation of serum iron

Principle

Iron in serum was estimated by Ramsay's (1958) dipyridyl method. In this, ferrous iron gives a pink colour with 2,2'- dipyridyl. A solution of dipyridyl in acetic acid is added to serum followed by a reducing agent. Heating in a boiling water bath followed by centrifugation and filtration removed all the proteins. The iron content was then measured at 520nm.

Reagents

1. (0.1%) 2, 2'- dipyridyl, in acetic acid (3%v/v).
2. 0.1M Sodium sulphite
3. Chloroform
4. Stock standard: Dissolved 0.498 g of ferrous sulphate $FeSO_4$. $7H_2O$ in distilled water. To this added 1 ml of concentrated sulphuric acid (Conc. H_2SO4). The solution contains 100 µg of iron per ml.
5. Working standard: Diluted 3 ml of the stock $FeSO_4.7H_2O$ solution to 100 ml with water. This contains 3 µg of iron per ml.

Procedure

To a known amount of the serum sample, 0.1M of sodium sulphite, 0.1% of 2,2' dipyridyl and distilled water was added. The test tubes were closed with a stopper. They were shaken well and was kept in a boiling water bath for 5 minutes. The tubes were then cooled. To this 1 ml of chloroform was added. All the tubes were stoppered and shaken vigorously for 30 seconds. The stoppers were then removed and tubes were centrifuged for 5 minutes at 3000 rpm. For standard, 2 ml of working ferrous sulphate standard solution and for blank, same amount of distilled water were taken. The pink colour obtained was read at 520 nm in a UV-VIS spectrophotometer.

d. Estimation of serum creatine kinase (E.C 2.7.3.2)

Principle

The method employed was described is that of Okinaka *et al.* (1961) with slight modifications. The increase in colour obtained by creatine with diacetyl and α-naphthol was measured at 520nm in which creatine phosphate was the substrate.

Reagents

1. 100mM Tris buffer of pH 7.4
2. 100mM HCl
3. 25mM Magnesium acetate solution
4. 12mM Creatine phosphate
5. 4mM Adenosine -5-diphosphate (ADP)
6. 150mM Cysteine
7. 30mM Parachloro mercuric benzoate (PCB)
8. Zinc sulphate solution: Dissolved 50 g of zinc sulphate in 1 litre of distilled water.
9. 150mM Barium hydroxide
10. Alkali solution: Mixed 60 g of NaOH and 128 g of anhydrous sodium carbonate in one litre of distilled water.
11. α-naphthol solution: 160 mg of α-naphthol was dissolved in 10 ml of alkali solution. The solution was freshly prepared before use. It was filtered to get a clear solution.
12. Diacetyl stock solution: 1.0 ml of diacetyl was first dissolved in small quantity of methanol. It was then made upto 100 ml with distilled water. The solution was kept in a brown bottle at 4^0C for further use.
13. Working solution of diacetyl: The stock solution was diluted into 1:20 with distilled water. The solution was prepared freshly before use.
14. Stock standard creatine solution (6 mM): 89.5 mg of creatine hydrate was dissolved in 100 ml of distilled water.
15. Working standard solution: Diluted 20 ml of the stock solution to 100 ml with distilled water.

Procedure:

To 0.2 ml of serum sample 0.2 ml of manganese solution, 0.1 ml of cysteine and 0.25 ml of creatine phosphate were added. The contents of the tubes were mixed well and were incubated at 37^0C for 5 minutes. After incubation 0.25 ml of ADP was added to the test tubes. The tubes were again subjected to incubation at 37^0C for 30 minutes. Then to each tube 0.5 ml of PCB, 0.5 ml of $Ba(OH)_2$ and 0.5 ml of zinc sulphate solution were added. The tubes were mixed well. It was then centrifuged at 4000 rpm for 5minutes. After centrifugation 1.0 ml of the supernatant was taken. To this supernatant, 2.5 ml of α-naphthol, 0.5 ml of diluted diacetyl solution and 6.0 ml of distilled water were added. The tubes were incubated at 37^0C for 1hr. It was then shaken intermittently for 15 minutes. The O.D was read at 520 nm against blank. A set of standards using creatine hydrate were also run in a similar manner. The enzyme activity thus obtained was expressed as units/L in serum.

e. Estimation of serum alanine transaminase (E.C 2.6.1.2)

Principle

Alanine transaminase activity in the serum was estimated by the method of of Reitman and Frankel (1957) in which pyruvate was measured by the reaction with 2,4-dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The color developed was then read at 520 nm.

Reagents

1. 0.1 M Phosphate buffer, pH 7.5
2. Substrate: 146 mg of α-ketoglutarate and 17.8 g of L-alanine was dissolved in 1 N NaOH with constant stirring. The pH was adjusted to 7.4. It was then made up to 1000 ml with phosphate buffer.
3. 2mM Standard pyruvate: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer of pH 7.5. 0.2 ml of standard pyruvate contained 0.4 μM of sodium pyruvate.
4. Dinitrophenyl hydrazine (DNPH) reagent, 1mmol/l: 200 mg/l in 1 mol/l HCL.
5. 0.4 N NaOH

Procedure

To 0.2 ml of the serum sample, 1.0 ml of the buffer substrate was added. It was incubated for 30 minutes at 37^0C. The reaction was arrested by adding 1.0 ml of DNPH. This serves as the 'Test'. To the control tubes, serum sample containing enzyme was added after arresting the reaction with 1.0 ml of DNPH. The tubes were kept at room temperature for 20 minutes. Then to each tube 10 ml of 0.4 N NaOH was added. A set of standards were also treated in a similar manner. The colour developed was then read at 520 nm. The enzyme activity thus obtained were then expressed as units/L in serum.

f. Estimation of serum aspartate transaminase (E.C 2.6.1.1)

Principle

Aspartate transaminase activity in the serum was estimated by the method of Reitman and Frankel (1957) in which oxaloacetate was measured by the reaction with 2,4-dinitrophenylhydrazine giving a brown coloured hydrazone after the addition of sodium hydroxide. The color developed was then read at 520 nm.

Reagents

1. 0.1 M Phosphate buffer, pH 7.5
2. Substrate: 146 mg of α-ketoglutarate and 13.3 g of aspartic acid was dissolved in 1 N NaOH with constant stirring. The pH was adjusted to 7.4. The solution was made up to 1000 ml with phosphate buffer.
3. Standard pyruvate, 2 mmol/l: 22 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. 0.2 ml of standard pyruvate contained 0.4 μM of sodium pyruvate.
4. Dinitrophenylhydrazine (DNPH) reagent, 1mmol/l: 200 mg/l of DNPH was dissolved in 1 mol/L HCl.
5. 0.4 N NaOH

Procedure

To 0.2 ml of the serum sample, 1.0 ml of the buffer substrate was added. It was then incubated for 60 minutes at 37^0C. This serves as the' Test'. The reaction was arrested by adding 1.0 ml of DNPH. To the control tubes, enzyme was added after arresting the reaction with 1.0 ml of DNPH. All the tubes were kept at room temperature for 20 minutes. Then to each tube 10 ml of 0.4 N NaOH was added. A set of standard pyruvate were also treated in a similar manner. The colour developed was read at 520 nm. The enzyme activity thus obtained was expressed as units/L in serum.

g. Estimation of serum alkaline phosphatase (E. C 3.1.3.1)

Principle

The method used for determining the activity of alkaline phosphatase was that of King and Armstrong (1934) with slight modifications in which disodium phenyl phosphate was hydrolysed with the liberation of phenol and inorganic phosphate. The liberated phenol was measured at 700 nm using Folin Ciocalteau reagent.

Reagents

1. Sodium carbonate- sodium bicarbonate buffer, 100mmol/L: 6.36 g of anhydrous sodium carbonate and 3.36 g of sodium bicarbonate was dissolved in distilled water and was made to a litre.
2. Disodium phenyl phosphate, 100mmol /L: 2.18 g of disodium phenyl phosphate was dissolved in water. It was allowed to boil, cooled and made to a litre. To this 1.0 ml of chloroform was added and was stored in the refrigerator.
3. Buffer – substrate: It was prepared by mixing equal volume of the above two solutions. This has a pH of 10.
4. Folin-Ciocalteau reagent: 1.0 ml of reagent was mixed with 2.0 ml of water(1:2 ratio)

5. 15% Sodium carbonate solution: 15 g of anhydrous sodium carbonate was dissolved in 100 ml of water.
6. Standard phenol solution, 1g/L: 1 g pure crystalline phenol was dissolved in 100 mmol/L HCl and was made to a litre with the acid.
7. Working standard solution: 100 ml dilute phenol reagent was added to 5.0 ml of stock standard. It was diluted to 500ml with water. This contained 10 µg of phenol/ml.

Procedure

4.0 ml of the buffer substrate was pipetted into a test tube and was incubated at 37^0C for 5 minutes. To this 0.2 ml of serum was added. It was then incubated further for exactly 15 minutes. This serves as the 'Test'. The tubes were removed and immediately 1.8 ml of diluted phenol reagent was added. At the same time a control was also kept containing 4.0 ml of buffer substrate and 0.2 ml of serum sample to which 1.8 ml phenol reagent was added immediately. All the tubes were mixed well and then centrifuged. 4.0 ml of the supernatant was taken. To this 2.0 ml of sodium carbonate was added. A set of standards and blank was also treated in the similar manner as that of 'Test'. The colour developed was then read at 700 nm. The enzyme activity thus obtained was then expressed as units/L in serum.

h. Estimation of serum total protein

Principle

Serum total protein was estimated by the method of Lowry *et al.* (1951). The blue color was developed by the reduction of the phosphomolybdic phosophotungstic components in the Folin-Ciocalteau reagent by the presence of amino acids tyrosine and tryptophan present in the protein sample. The colour developed by the reaction of protein with alkaline cupric tartrate was measured at 660 nm.

Reagents

1. 2% Sodium carbonate in 0.1 N NaOH (Reagent A)
2. 0.5% Copper sulphate in 1% potassium sodium tartrate (Reagent B)
3. Alkaline copper reagent: 50 ml of A and 1.0 ml of B were mixed prior to use (Reagent C).
4. Folin-Ciocalteau reagent: 1 part of reagent was mixed with 2 parts of water (1:2 ratio)
5. Stock standard: 50 mg of bovine serum albumin was weighed. It was then made up to 50 ml in a standard flask with distilled water.
6. Working standard: 10 ml of the stock was diluted to 50 ml with distilled water. 1.0 ml of this solution contains 200 µg of protein.

Procedure

A set of working standard solution ranging from 0.2 to 1.0 ml was pipetted out into a test tube. The volume in all the tubes were made up to 1.0 ml with distilled water. 5.0 ml of alkaline copper reagent was added to all of the test tubes. The contents in the tubes were mixed well and was allowed to stand for 10 minutes. Then 0.5 ml of Folin-Ciocalteau reagent was added, mixed well and were incubated at room temperature for 30 minutes. A reagent blank was also prepared in the similar manner. After 30 minutes, the blue colour developed was read at 660 nm. Serum samples were also treated in the similar manner. The results obtained were expressed as g/dl in serum.

5.1C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l concentrations of ethanol on *O. mossambicus* exposed for 21 days with a periodical sampling for 7 days exhibited marked differences in serum iron, creatine kinase, alanine transaminase, aspartate transaminase, alkaline phosphatase and total protein as shown in the tables 5.1.1 to 5.1.9 and in figures 5.1.3 to 5.1.8. Results were statistically analyzed by Two way – ANOVA (Analysis of Variance) followed by Dunnett's method.

Table 5.1.1 Effect of exposure to different concentrations of ethanol for 7 days on serum parameters of *O. mossambicus*.

Serum Parameters	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Iron (µg/dl)	48.77± 0.4762	51.85± 0.8105	66.59± 0.4543	79.60± 0.5872
Creatine Kinase (IU/L)	13.82± 0.5616	54.06± 0.3675	87.40± 1.0574	108.53± 0.9475
Alanine Transaminase (U/L)	12.26± 0.2872	13.50± 0.3058	14.44± 0.2510	17.38± 0.2278
Aspartate Transaminase (U/L)	17.98± 0.2188	23.46± 0.2785	54.10± 0.4682	75.94± 0.5379
Alkaline Phosphatase (U/L)	22.04± 0.1823	23.24± 0.3089	25.38± 0.3646	27.34± 0.4137
Total Protein g/dl)	1.533± 0.0393	1.483± 0.0393	1.280± 0.0363	1.095± 0.0565

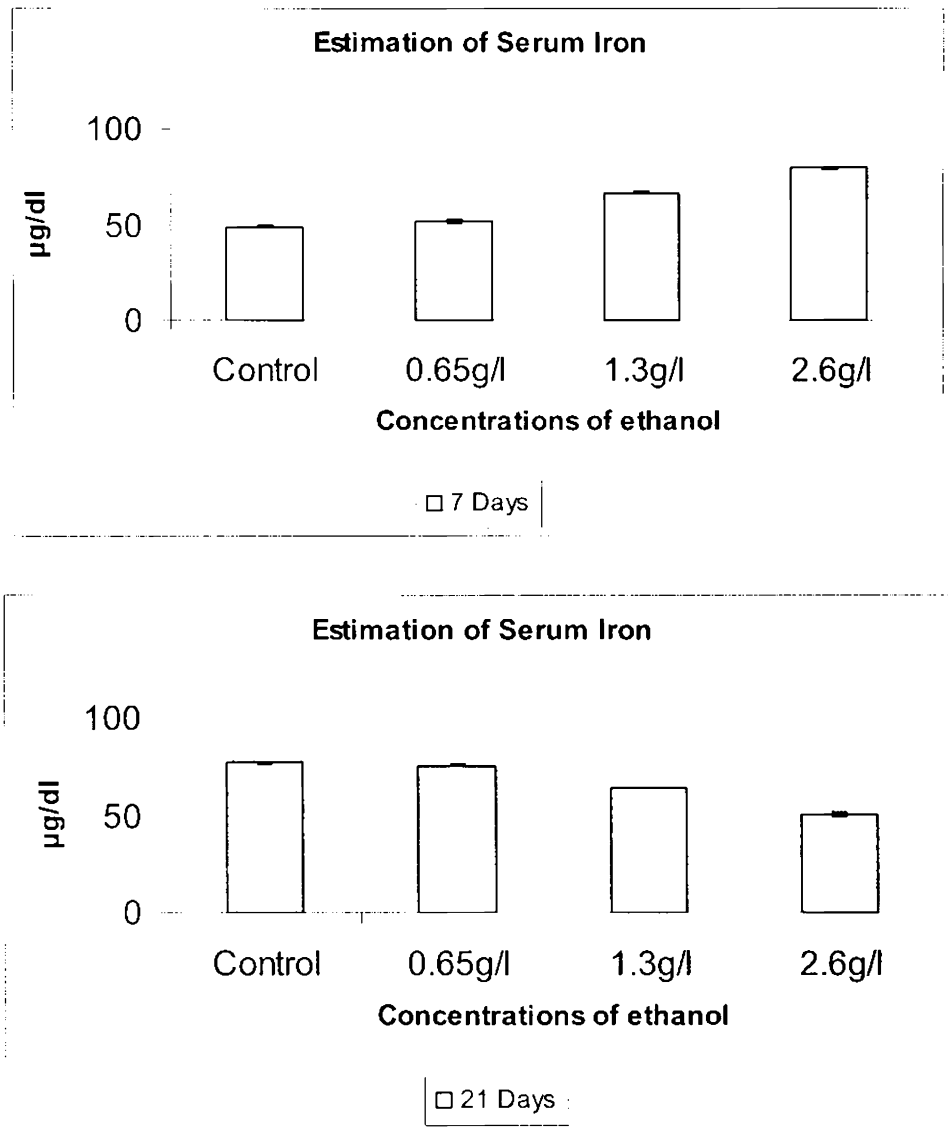
Average of six values in each group ± SD of six observations.

Table 5.1.2 Effect of exposure to different concentrations of ethanol for 21 days on serum parameters of *O. mossambicus*.

Serum Parameters	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Iron (µg/dl)	77.34± 0.6033	75.59± 0.4565	64.06± 0.2435	50.66± 0.6713
Creatine Kinase (IU/L)	21.79± 0.3328	24.93± 0.2342	36.84± 0.4145	41.72± 0.9253
Alanine Transaminase (U/L)	13.20± 0.3251	15.09± 0.3344	16.47± 0.3494	19.93± 0.4597
Aspartate Transaminase (U/L)	21.37± 0.2922	41.87± 0.3281	44.23± 0.3184	55.25± 0.4197
Alkaline Phosphatase (U/L)	23.12± 0.1799	25.03± 0.4077	26.26± 0.1632	27.04± 0.5450
Total Protein g/dl)	2.225± 0.0274	1.837± 0.0339	1.630± 0.0261	1.548± 0.0349

Average of six values in each group ± SD of six observations.

Figure 5.1.3 Levels of iron in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Significant differences ($P<0.001$) were observed in the serum iron level of *O. mossambicus* exposed to 7 and 21 days (Figure 5.1.3). Statistical approach employing ANOVA corroborates this as can be inferred from the table below (Table 5.1.3a).

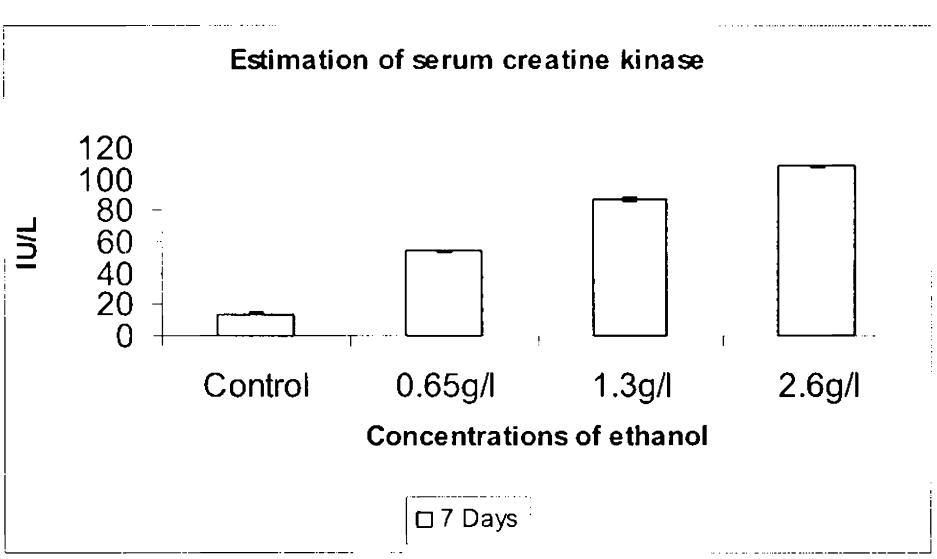
Table 5.1.3a ANOVA Table for serum iron

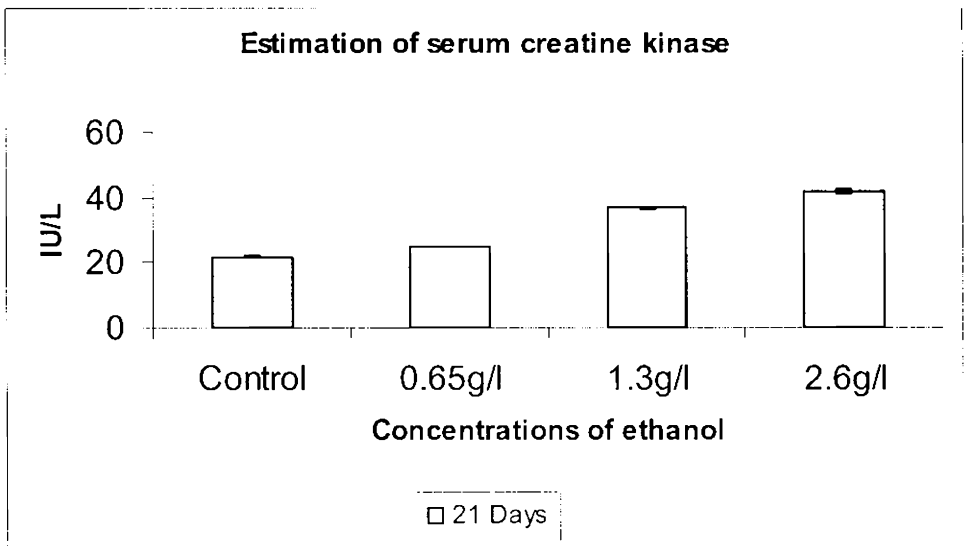
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	325.365	1	325.365	1049.305	0.000
Between Concentrations	43.371	3	14.457	46.624	0.000
Days of Exposure × Concentration	6345.777	3	2115.259	6821.738	0.000
Error	12.403	40	0.310		
Total	6726.916	47			

df – degrees of freedom

Two Factor ANOVA table revealed that serum iron levels varied significantly ($P<0.001$) between days of exposure. Between concentrations there was a significant difference ($P<0.001$). Also while taking into consideration the effects of both the days as well as concentrations (Interaction) significant difference ($P<0.001$) was observed.

Figure 5.1.4 Levels of creatine kinase in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant increase (P< 0.001) in serum creatine kinase level was noted in *O. mossambicus* exposed to the three sub lethal concentrations of ethanol as compared to control group (Figure 5.1.4). Investigations using ANOVA substantiates the above statement and the results are shown below (Table 5.1.4a).

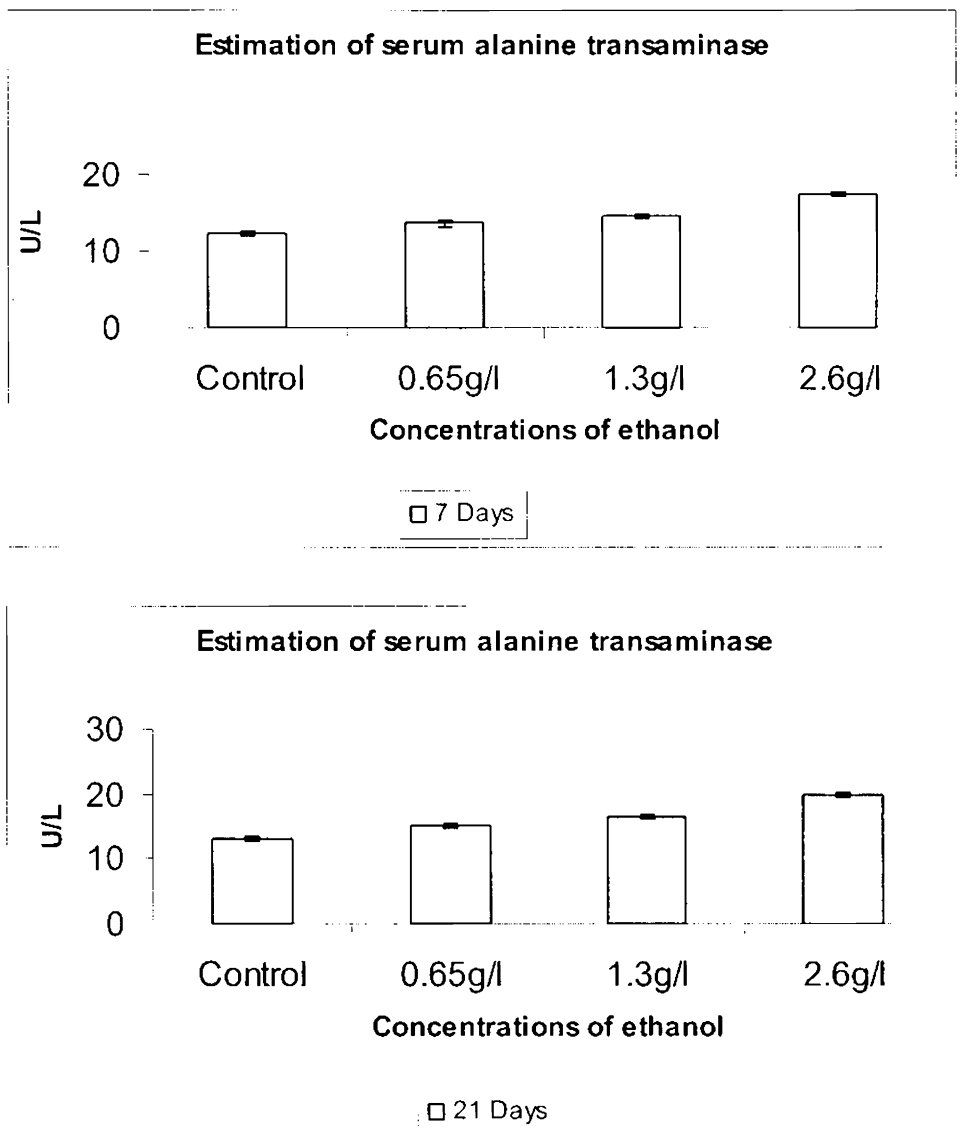
Table 5.1.4 a ANOVA Table for serum creatine kinase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	14391.189	1	14391.189	31458.071	0.000
Between Concentrations	23010.683	3	7670.228	16766.548	0.000
Days of Exposure × Concentration	9402.434	3	3134.145	6851.008	0.000
Error	18.299	40	0.457		
Total	46822.605	47			

df-degrees of freedom

Two Factor ANOVA table indicated that serum creatine kinase levels varied significantly (P<0.001) between days. Between concentrations a marked significant difference (P<0.001) was noted. After considering the interaction effect of both the days as well as concentrations significant difference (P<0.001) was seen.

Figure 5.1.5 Levels of alanine transaminase in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



A Significant increase ($P<0.001$) was observed in the serum alanine transaminase level of *O. mossambicus* exposed to 7 and 21 days (Figure 5.1.5). Statistical approach employing ANOVA corroborates this as can be inferred from the table below (Table 5.1.5a).

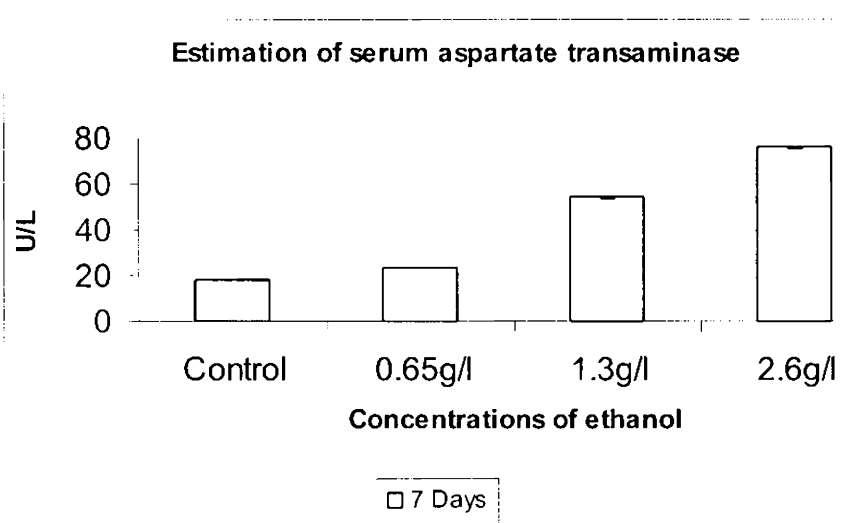
Table 5.1.5 a ANOVA Table for serum alanine transaminase

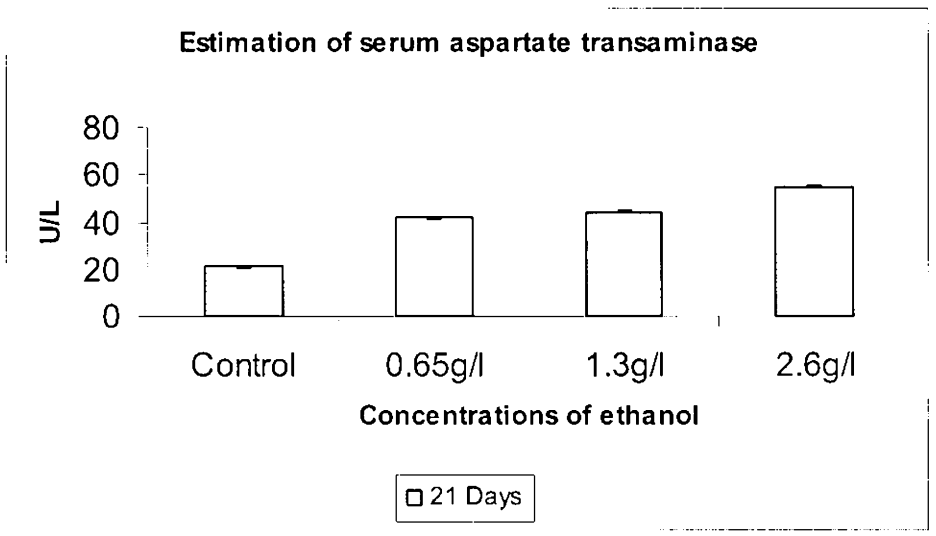
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	37.914	1	37.914	360.311	0.000
Between Concentrations	227.036	3	75.679	719.204	0.000
Days of Exposure × Concentration	4.254	3	1.418	13.477	0.000
Error	4.209	40	0.105		
Total	273.414	47			

df-degrees of freedom

Statistical analysis done by using Two Factor ANOVA revealed that serum alanine transaminase levels varied significantly between days ($P<0.001$). It was noted that between concentrations significant difference was $P<0.001$. It was concluded that by taking into consideration both the days as well as concentrations (Interaction) significant difference ($P<0.001$) was observed.

Figure 5.1.6 Levels of aspartate transaminase in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





O. mossambicus treated with various ethanol concentrations exhibited a significant ($P<0.001$) increase in serum aspartate transaminase level when compared to the control groups on both periods of exposure (Figure 5.1.6). ANOVA was carried out to ascertain the statement and the table is shown below (Table 5.1.6a).

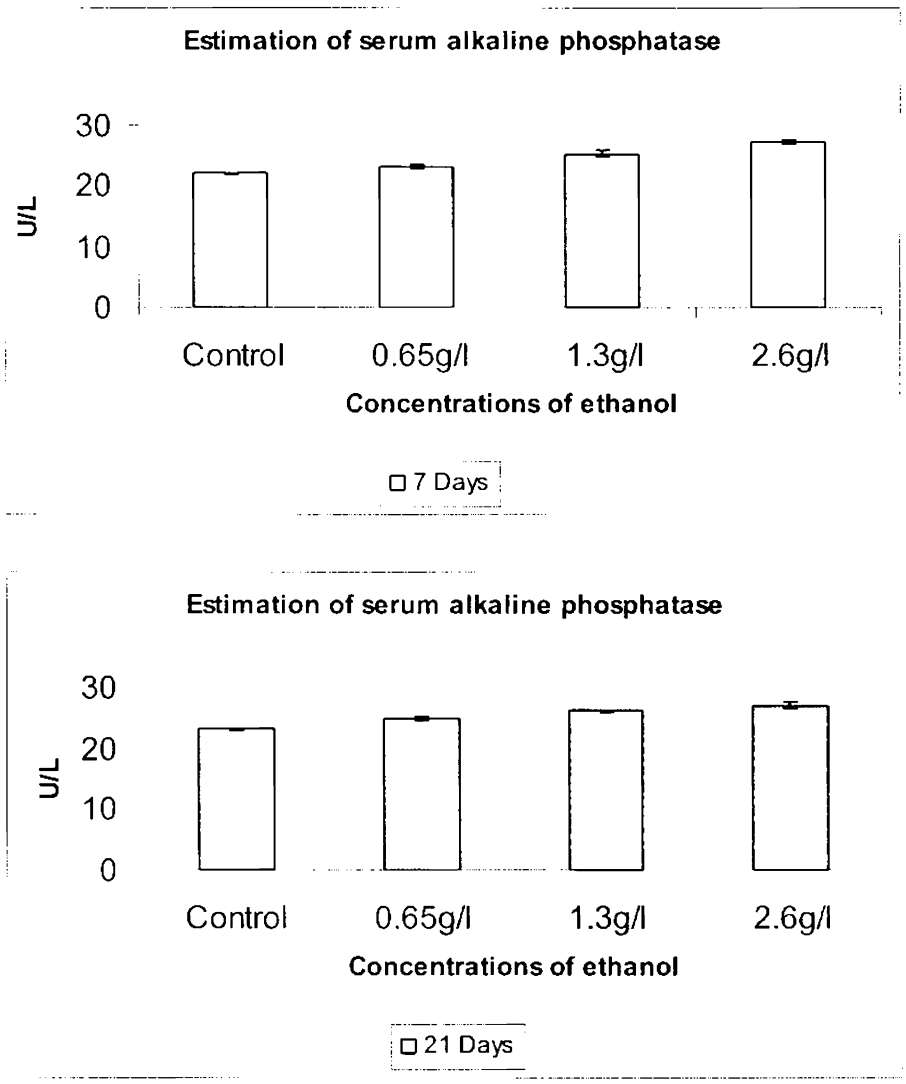
Table 5.1.6a ANOVA Table for serum aspartate transaminase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	57.466	1	57.466	416.222	0.000
Between Concentrations	14321.033	3	4773.678	34575.582	0.000
Days of Exposure × Concentration	2570.061	3	856.687	6204.953	0.000
Error	5.523	40	0.138		
Total	16954.083	47			

df-degrees of freedom

By using Two Factor ANOVA it was noted that serum aspartate transaminase level varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 5.1.7 Levels of alkaline phosphatase in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Significant ($P<0.001$) increase was observed in different sub lethal concentrations of the treatment group when compared to control group of *O. mossambicus* during exposure periods of 7 and 21 days (Figure 5.1.7). Statistical approach using ANOVA substantiates the above statement (Table 5.1.7a).

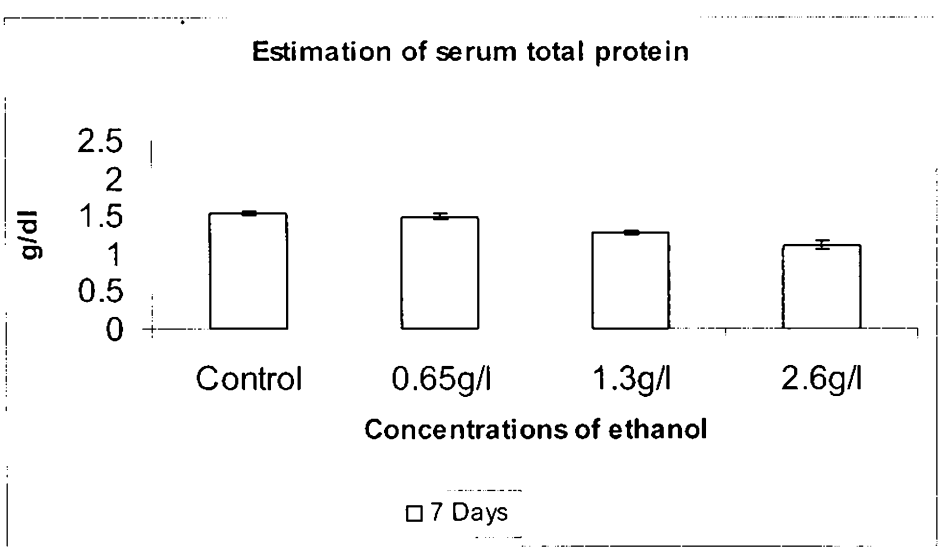
Table 5.1.7 a ANOVA Table for serum alkaline phosphatase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	8.858	1	8.858	74.212	0.000
Between Concentrations	144.737	3	48.246	404.201	0.000
Days of Exposure * Concentration	6.736	3	2.245	18.812	0.000
Error	4.774	40	0.119		
Total	165.105	47			

df-degrees of freedom

Two Factor ANOVA table revealed that in the case of serum alkaline phosphatase levels between days, no significant difference was observed. Between concentrations there was a significant difference ($P<0.001$). When taken into consideration the effects of both days as well as concentrations (interaction) significant difference ($P<0.001$) was observed.

Figure 5.1.8 Levels of protein in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



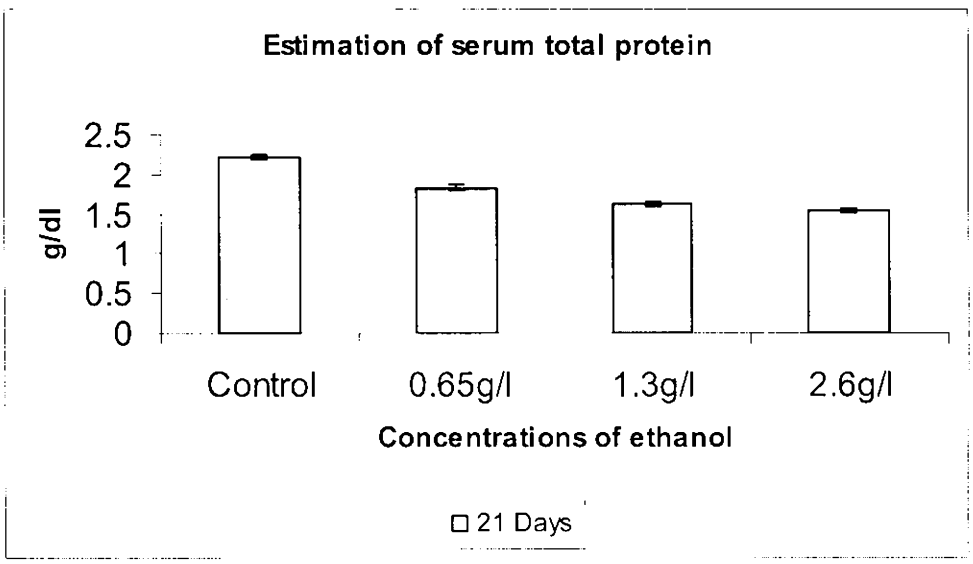


Figure 5.1.8 depicts significant decrease ($P<0.001$) in the serum protein level in *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure period (Figure 5.1.8). Analysis carried out by applying ANOVA supports the above statement (Table 5.1.8a).

Table 5.1.8a ANOVA Table for serum total protein

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	2.562	1	2.562	1798.598	0.000
Between Concentrations	2.139	3	0.713	500.518	0.000
Days of Exposure × Concentration	0.232	3	0.077	54.173	0.000
Error	0.057	40	0.001		
Total	4.990	47			

df-degrees of freedom

By using Two Factor ANOVA it was noted that serum protein levels varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Table 5.1.9 Multiple Comparison Test

Subsequent comparisons by multiple comparison tests using Dunnett's is shown below.

	Groups	Serum Iron	Serum CK	Serum ALT	Serum AST	Serum ALP	Serum Total Protein
Dunnett	Control Vs 0.65g/l	0.016[b]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = $P < 0.001$, b = $P < 0.05$ and not significant at c.

Subsequent comparisons by multiple comparison test using Dunnett's method is shown above (Table 5.1.9)

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. In the case of serum iron values with respect to control, significant difference ($P<0.05$) was observed at 0.65 g/l. Also while considering 1.3 g/l and 2.6g/l significant difference ($P<0.001$) was observed with respect to control. While considering all other serum parameters such as creatine kinase, aspartate transaminase, alanine transaminase, alkaline phosphatase and total protein levels significant difference ($P<0.001$) was observed in all the three concentrations with respect to control.

5.1D DISCUSSION

The effects of xenobiotic contamination in an ecosystem can be estimated through analysis of biochemical changes in organisms inhabiting that region (Brewer *et al.*, 2001; Norris *et al.*, 2000; Tuvikene *et al.*, 1996). Fish species are widely used to monitor variation in environmental levels of anthropogenic pollutants. Kanbak *et al.* (2001) have stated ethanol as a direct systemic toxin that produces injury to all tissues, depending on the dose and duration of exposure. The degree of injury varies among organ systems. Several specific enzymes have been proposed for monitoring purposes of water pollution (Agradi *et al.*, 2000). An

attempt has been made in the present study to determine the ethanol induced alterations in serum of the fresh water fish *O. mossambicus*.

Iron is an important bio-catalyst of oxidation-reduction reactions in the cell and is essential for life. The presence of iron in cytochromes, catalase, hydroxylase, peroxidases, saturases, lipoxygenases and cyclo oxygenases suggest that iron has an important role in various metabolic events related to lipids, such as the oxidative degradation of fatty acids and in the synthesis of unsaturated fatty acids, plasminogens and prostaglandins. Iron was chosen as a focus because ethanol exposure can lead to an increase in the concentration of free iron (Mendelson and Jiner, 1994). Iron is an essential micronutrient of teleost fish and is an integral component of proteins involved in cellular respiration and oxygen transfer. Ethanol brings about disturbances in iron metabolism. In the present study a significant increase (Fig 5.1.3a) in serum iron was observed when exposed to 7 days which was due to increased iron absorption. The findings of Charlton *et al.* (1964) and Sanchez *et al.* (1988) support the present observations. When fishes were exposed for 21 days a significant decrease ($P<0.001$) (Fig 5.1.3a) in the serum iron value was noted. The decreased iron bioavailability may be one of the reasons for the alterations in the iron content of the body. Decreased iron absorption was probably the main cause of iron body depletion. The present finding was supported by Lundvall *et al.* (1969) who too observed similar increase in the serum iron levels under chronic conditions of pollution.

Serum enzymes have close relationship to a particular organ or tissue and possess high degree of specificity. These enzymes are intracellular and are present in small amounts in the blood of healthy animals. But, when there is an increase of cell breakdown due to damage of tissues, caused either by disease or pollutants they escape in greater quantities into the blood with consequent increase in their activities in serum. The increased levels of these enzymes in blood have also been reported due to their leakage from the toxin-affected tissues to the circulating fluid (Chenoweth and Ellman, 1957; Rees and Sinha, 1960). Alanine aminotransferase (ALT, formerly SGPT), aspartate aminotransferase (AST, formerly SGOT), creatine kinase (CK) and alkaline phosphatase (ALP) were selected as relevant enzymes for

evaluating liver intoxication (Krajnovic and Krajnovic, 1992). They are used as sensitive biomarkers in ecotoxicology where they provide an early warning of potentially hazardous alterations seen in contaminated aquatic organisms (Levesque *et al.*, 2002; Vaglio and Landriscina, 1999; Dela Torre *et al.*, 2000). The measurement of phosphatase and transaminase activities in the circulating fluid is frequently used as a diagnostic tool in water pollution studies (Palanivelu *et al.*, 2005). The role of blood enzymes in monitoring and detecting stress or disease has led to a growing concern in using them as biochemical indicators to trace environmental pollutants. An increase in serum creatine kinase values observed when *O. mossambicus* was subjected to 7 days of exposure to different sub lethal concentrations of ethanol was due to injury occurred in the cardiac tissues which in turn elevated the serum creatine kinase activities. Increase in CK during immediate exposure was supported by Kurup *et al.* (1991) who also found similar change in the serum of rats when treated with ethanol for 7 days. Muralidhar *et al.* (2004) also observed similar increase in the serum CK values in mice on injecting them with ethanol during acute conditions. When exposed for 21 days significant increase was observed which also refers to the increased cardiac injury resulting in simultaneous elevation of creatine kinase levels. Similar findings have been made by Anbarasi *et al.* (2005) who also observed an increase in the creatine kinase activity in the serum of rats upon chronic exposure to cigarette smoke. In the present study a significant increase ($P<0.001$) (Fig 5.1.5a and 5.1.6a) in serum ALT and AST levels was observed when *O. mossambicus* was subjected to different sub lethal concentrations of ethanol during 7 days of exposure. This explains that during hepatobiliary disorder, amino acids are released from damaged tissues. In order to metabolize these amino acids, the process of transmutation gets enhanced leading to increased activity of the related enzymes AST and ALT. The increase in serum AST and ALT during immediate exposure was cited by Nassr and Abdel (2007) in his studies on *Oreochromis aureus* subjected to phenol exposure. The same findings were recorded by Kumari *et al.* (1993) who also found increased ALT and AST activities in the serum samples of 30 cases of pediatric protein energy malnutrition. Prolonged exposure also exhibited significant increase in serum ALT and AST which is related to increased damage brought to the tissues such as liver, kidney and

heart in the state of stress influenced by ethanol resulting in the liberation of these transaminases into the circulation resulting in impairment of fish metabolism. The findings of Singh and Reddy (1990) prove that similar changes occur in *Heteropneustes fossilis* when exposed to copper sulphate for prolonged exposure. The findings by Joy *et al.* (1993) also corroborates the present findings. A significant increase in serum ALP activity observed during exposure to ethanol for 7 days has been related to tissue damage. Observations made by Karan *et al.* (1998) support the present finding as they too observed similar increase in ALP activity in the serum of *Cyprinus carpio* when exposed to copper. On exposure to different sub lethal concentrations of ethanol for 21 days, *O. mossambicus* showed significant increase in serum ALP activity which indicates severe liver destruction brought about by ethanol leading to the release of ALP into the blood. The increase in the serum enzyme values noted by these authors was directly proportional to the degree of cellular damage. Increase in serum ALP during prolonged exposure to ethanol was supported by Diana and Marina (2004).

A significant decrease in total serum protein was observed on exposure to ethanol for 7 days. Decrease in protein was mainly due to the stress felt in fish which led to the degradation of protein into free amino acids. Under stress protein is likely to undergo hydrolysis and oxidation through TCA cycle to meet the increased demand for energy. Similar observation has been cited by Ahmed *et al.* (2002) in the serum of rat on exposure to ethanol. The observations made by Mirsky and Pauling (1936) stress that ethanol denatures proteins by breaking hydrogen bonds. In the present study a significant decrease in serum total protein was observed when *O. mossambicus* was exposed to different sub lethal concentrations of ethanol for 21 days. This in turn is related to decrease in body weight which simultaneously brings about deficiency of protein. The decrease in protein content along with the decrease in liver weights suggest increased utilization of protein reserves of liver during stress induced starvation and/or retardation of protein synthesis by toxicants. The decrease in protein content with simultaneous decrease in DNA or RNA, or both DNA and RNA clearly suggests the inhibitory effect of effluent at transcription /translation levels (Kaur and Kaur, 2005). This suggests a good indication of health status of fish. This observation was supported by Eisenstein and Harper (1991). Decrease in

serum total protein during prolonged exposure was supported by Paulov and Demers (1971) who obtained similar results in young ducklings treated with ethanol.

Thus, it can be concluded that ethanol has serious consequences on serum parameters in *O. mossambicus*.

5.2 EFFECT OF ETHANOL ON SELECTED SERUM PARAMETERS OF *OREOCHROMIS MOSSAMBICUS* (PETERS): STUDIES USING AUTO ANALYZER.

Contents

5.2A Introduction
5.2B Materials and Methods
5.2B.1 Estimation of serum parameters using immunoassay analyzer
a. Estimation of serum cortisol
b. Estimation of serum ferritin
c. Estimation of serum vitamin B12
d. Estimation of serum folic acid
5.2C Results
5.2D Discussion

5.2A Introduction

Stress is defined as the response of the cell, or organism, to any demand placed on it such that it causes an extension of a physiological state beyond the normal resting state (Barton, 1997). Environmental condition has been increasingly degenerated due to industrial development and other human activities. Increasing pace of industrialization in public and private sectors along with urbanization, population explosion and green revolution, clearly reflects in varying degrees of pollution of water, soil and air. Industrial development has generally been equated with ecological degradation which leads to environmental pollution. The past decade has seen a flood of information on the metabolic and physiological effects of stress in fish (Barton and Iwama, 1991; Gamperl *et al.*, 1994; Iwama *et al.*, 1998; Pickering and Pottinger, 1995; Wendelaar Bonga, 1997). The widespread usage of ethanol ultimately pollutes the aquatic environment thereby affecting the aquatic fauna mainly fishes which constitutes the major economy of the country and are valuable sources of protein. Increasing awareness of the adverse effects of anthropogenic activities and pollution on aquatic environment has focused interest on health of fish populations and possibilities to utilize these health parameters for assessing the

quality of aquatic environment (Henry *et al.*, 2004). Previous studies done by (Barnhart, 1969; Smith and Ramos, 1980; Warner *et al.*, 1978, 1979) have demonstrated that automated chemistry analysis systems designed primarily for human clinical samples could provide accurate measures of comparable components found in sera of other fish species if the samples were properly handled. Automated analysis has also been used to determine some serum parameters for channel catfish (Kirk, 1974; Limsuwan *et al.*, 1983; Warner and Williams, 1977). Stress in fish has been shown to cause a primary response, involving neuro - hormonal stimulation, resulting in an increase in corticosteroid and catecholamine secretions. One of the most commonly measured indicators of stress in fish is the concentration of the major circulating corticosteroid, cortisol (hydrocortisone). Cortisol hormone in fish has been identified as a metabolic hormone whose action is multi-faceted (Vijayan *et al.*, 1994). It is also considered as an important stress hormone produced in fish (Mommsen *et al.*, 1999). The catabolic hormones include corticosteroids and catecholamines. Cortisol is the predominant corticosteroid in most of the teleost group (Henderson and Garland, 1980). The catabolic action of cortisol is responsible for the mobilization of energy reserves (gluconeogenesis) and lipolysis (Leach and Taylar, 1982; Sheridan, 1986). Vitamin B12 functions as a coenzyme in a number of metabolic reactions. A specific function is to act in concert with folic acid in the transfer of single-carbon units, such as methylation of uracil to form thymine in DNA synthesis and in methyl transfer in methionine synthesis. Vitamin B12 is necessary for normal growth, maturation of erythrocytes, and healthy nervous tissue. A vitamin B12 deficiency could cause a folic acid deficiency because it is necessary for conversion of tetrahydrofolic acid to its coenzyme form. Intrinsic factor, a mucoprotein in the digestive tract, is necessary for proper absorption of B12. It has been found in the gut of most animals. Folate is an essential micronutrient in mammals that must be obtained from exogenous sources via intestinal absorption. Adequate folate intake is vital for cell division and homeostasis because folate coenzymes play important roles in nucleic acid synthesis, methionine regeneration, and in shuttling oxidation and reduction of one-carbon units required for normal metabolism and regulation (Wagner, 1995). Ferritin is the storage form of iron and high levels of ferritin are always associated with Coronary artery disease (Salonen *et*

al., 1992). The concentration of ferritin in the serum is directly proportional to the levels of body iron stores. The body iron status is related to the concentration of the ferritin, its storage form and transferring, the transport form. The present study was carried out to assess the effect of cortisol, ferritin, folic acid and vitamin B 12 on serum of *O. mossambicus* using automated analyzer since it can be measured easily and accurately.

5.2B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5. Preparation of serum samples for experimental studies remains the same as described in Chapter 5.1B. Serum samples were analyzed using Roche Elecys 2010 and Modular Analytics E170 (Elecsys module) immunoassay analyzer.

5.2B.1 Estimation of serum parameters using immunoassay analyzer

The following parameters were studied using immunoassay analyzer.

a. Estimation of serum cortisol

Principle

The determination of cortisol in serum was done by the Electro Chemiluminescence immunoassay (ECLIA). The method employed was that of competition test principle using a polyclonal antibody which is specifically directed against cortisol. Endogeneous cortisol present in the sample has been liberated from the binding protein with danazol which competes with the exogeneous cortisol derivative in the test which has been labeled with ruthenium complex for the binding sites on the biotinylated antibody.

Reagents

Elecsys cortisol reagent kit, cat. No. 11875116 was used for the assay.

1. Streptavidin coated microparticles, 0.72 mg/ml; binding capacity: 470 ng biotin/mg microparticles.

2. Anti-cortisol-Ab-biotin, Biotinylated polyclonal anti-cortisol antibody (ovine) 90 ng/ml; MES buffer 100 mmol/L pH 6.0
3. Cortisol derivative (Synthetic) labeled with ruthenium complex 25 ng/ml; danazol 20 µg/ml; MES buffer 100 mmol/ pH 6.0

Procedure

To 20 µl of serum sample, cortisol-specific biotinylated antibody and a ruthenium complex labeled cortisol derivative were added. It was incubated at 37^0C for 9 minutes. Streptavidin-coated microparticles were added and it was incubated at 37^0C for 9 minutes. This forms complex which gets bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was then aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell. Voltage was applied to the electrode which induced chemiluminescent emission. It was measured by a photomultiplier. Results thus obtained were determined via a calibration curve which was instrument specific generated by a 2-point calibration and a master curve provided via the reagent barcode. The results obtained were then expressed as µg/dl.

b. Estimation of Serum folate

Principle

Folate activity in serum was estimated by the method of Electro Chemiluminescence immunoassay (ECLIA). The method employs a competition test principle using natural folate binding protein (FBP) specific for folate. Folate present in the sample competes with the added folate (labeled with biotin) for the binding sites on FBP (labeled with ruthenium complex).

Reagents

Employs Elecsys Folate II reagent kit, cat. No. 03253678 was used for the assay.

1. Monothioglycerol 53.3 g/L, pH 5.5

2. Sodium hydroxide 37 g/L
3. Streptavidin-coated microparticles, 0.72 mg/ml, binding capacity: 470 ng biotin/mg microparticles
4. Ruthenium labeled folate binding protein 50 µg/L, human serum albumin; borate/phosphate/citrate buffer; pH 5.5
5. Biotinylated folate 18 µg/L; biotin 120 µg/L; human serum albumin; borate buffer, pH 9.0

Procedure

To 30 µl of serum sample, monothioglycerol and sodium hydroxide were added. It was incubated at 37^0C for 9 minutes. After incubation the pretreated sample was then added to the ruthenium labeled folate binding protein. It was then incubated at 37^0C for 9 minutes, which resulted in the formation of a folate complex. Streptavidin – coated microparticles and folate labeled with biotin were added. It was then again incubated at 37^0C for 9 minutes. This resulted in the formation of a ruthenium labeled folate binding protein-folate biotin complex. The entire complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were removed with ProCell. Voltage was applied to the electrode which induces chemiluminescent emission. It was then measured by a photomultiplier. Readings thus obtained were calculated via a calibration curve which was instrument specific generated by a 2-point calibration and a master curve provided via the reagent barcode. The results obtained were expressed as ng/ml.

c. Estimation of serum vitamin B12

Principle:

Vitamin B12 in serum was determined by Chemiluminescent immuno assay using the Access Immunoassay systems REF 33000. The method employed was that of competitive binding immuno enzymatic assay in which B12 binding proteins gets denatured and converts all forms of vitamin B12 to the cyanocobalamin form.

Reagents

1. Paramagnetic particles coated with goat anti-mouse Ig G: Mouse monoclonal anti-intrinsic factor complexes, TRIS buffered saline, surfactant, bovine serum albumin (BSA), <0.1% sodium azide and 0.1% proclin 300
2. Borate buffer with surfactant, cobinamide and <0.1% sodium azide.
3. Porcine intrinsic factor– alkaline phosphatase (bovine) conjugate in TRIS buffered saline, surfactant, human serum albumin (HAS), <0.1% sodium azide, and 0.25% proclin 300
4. 0.5N Sodium hydroxide solution with 0.005% potassium cyanide (KCN)
5. 0.02% acetic acid solution with dithiothreitol (DTT)

Procedure

To 45 µl of the serum sample alkaline potassium cyanide and ditiothreitol was added. It was subjected to neutralization. After neutralization, intrinsic factor -- alkaline phosphatase conjugate and paramagnetic particles coated with goat anti-mouse Ig G: mouse monoclonal anti-intrinsic factor was added to the sample. It was then incubated at 37^0C for 10 minutes. After incubation in a reaction vessel, materials bound to the solid phase were held in a magnetic field while unbound materials were washed away. Then the chemiluminescent substrate Lumi-Phos 530 was added to the vessel and the light generated by the reaction was measured with a luminometer. The photons produced were inversely proportional to the concentration of vitamin B12 in the sample. The amount of analyte present in the sample was determined by means of a stored, multi-point calibration curve which was expressed in pg/ml.

d. Estimation of Serum ferritin

Principle

Ferritin in serum was estimated by the method of electro chemi luminescence immunoassay (ECLIA). The method employed was that of a sandwich principle.

Reagents

Elecys Ferritin reagent kit, Cat no. 03737551 was used for the assay

1. Streptavidin-coated microparticles 0.72 mg/ml: binding capacity: 470 ng biotin/mg microparticles.
2. Biotinylated monoclonal anti-ferritin antibody 3 mg/L; phosphate buffer 100 mmol/L, pH 7.2
3. Monoclonal anti-ferritin antibody labeled with ruthenium complex 6.0 mg/L; phosphate buffer 100 mmol/L, pH 7.2.

Procedure:

To 10 µl of the serum sample, a biotinylated monoclonal ferritin-specific antibody, and a monoclonal ferritin-specific antibody labeled with a ruthenium complex were added which in turn forms a sandwich complex. It was subjected to incubation at 37^0C for 9 minutes. After incubation streptavidin-coated micro particles were added. It was then incubated at 37^0C for 9 minutes. This forms a complex which gets bound to the solid phase. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were removed with the procell. Voltage was applied to the electrode which then induced chemiluminescent emission. It was measured by a photomultiplier. Results thus obtained were determined via a calibration curve which was instrument specific generated by a 2-point calibration and a master curve provided via the reagent barcode. The results obtained were expressed as ng/ml.

5.2C RESULTS

Table 5.2.1 Effect of exposure to different concentrations of ethanol for 7 days on serum parameters of *O. mossambicus*.

Serum Parameters	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Ferritin (ng/ml)	0.143± 0.0314	0.227± 0.0308	0.332± 0.0293	0.430± 0.0253
Cortisol (μg/dl)	5.618± 0.3143	8.620± 0.2740	9.542± 0.2548	10.73± 0.2397
Folate (ng/ml)	11.39± 0.2956	7.613± 0.2376	6.430± 0.1515	6.038± 0.1347
Vitamin B12 (pg/ml)	7.368± 0.0027	7.411± 0.0025	7.421± 0.0011	7.464± 0.0009

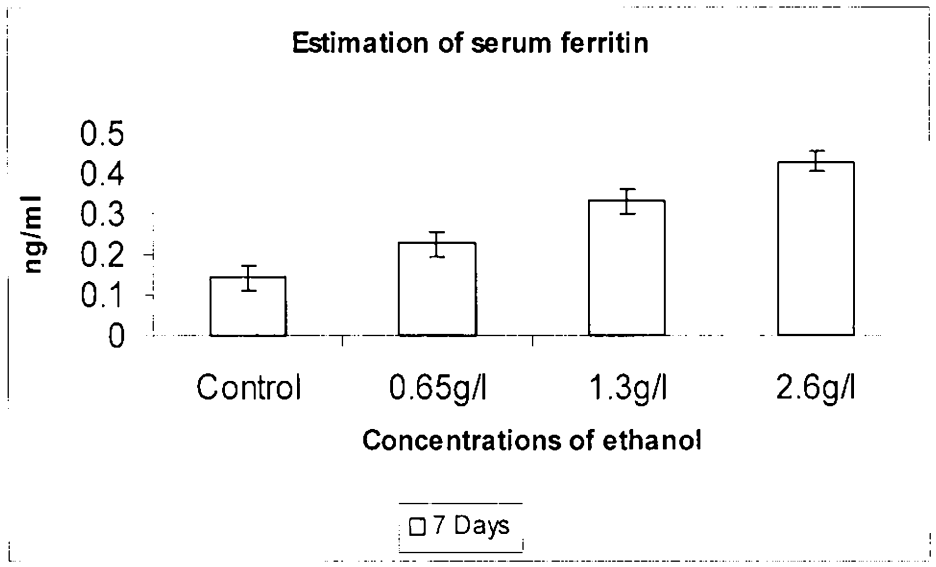
Average of six values in each group ± SD of six observations.

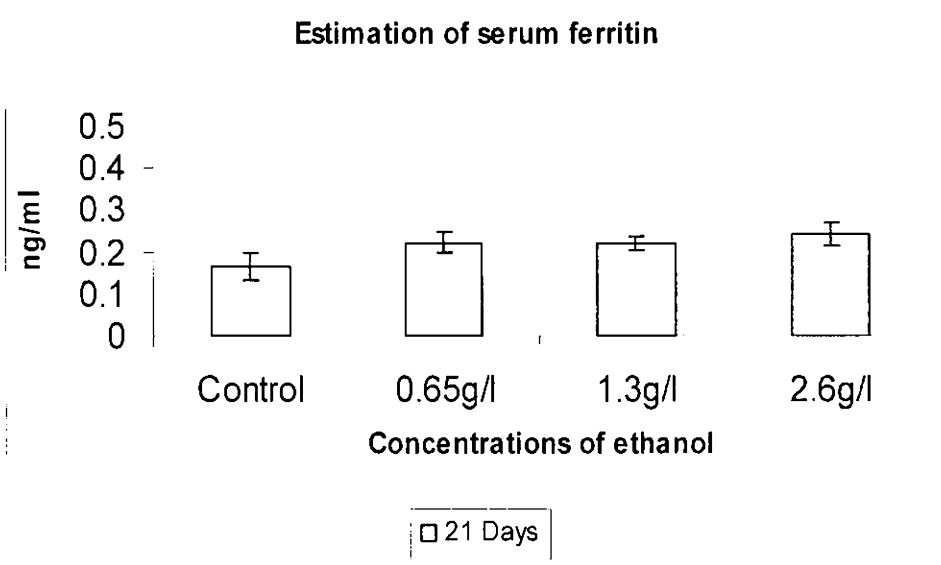
Table 5.2.2 Effect of exposure to different concentrations of ethanol for 21 days on serum parameters of *O. mossambicus*.

Serum Parameters	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Ferritin (ng/ml)	0.165± 0.0327	0.222± 0.0263	0.220± 0.0141	0.243± 0.0273
Cortisol (μg/dl)	6.500± 0.3357	7.678± 0.2416	9.537± 0.1314	11.08± 0.3498
Folate (ng/ml)	13.91± 0.2303	29.11± 0.2146	30.34± 0.2439	32.29± 0.2015
Vitamin B12 (pg/ml)	7.423± 0.0026	7.451± 0.0018	7.455± 0.0007	7.458± 0.0011

Average of six values in each group ± SD of six observations.

Figure 5.2.3 Levels of ferritin in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





O. mossambicus exposed to varying sub lethal concentration of ethanol exhibited marked significant ($P<0.001$) (Figure 5.2.3) increase in the serum ferritin value. The ANOVA table mentioned below (Table 5.2.3a) justifies this conclusion.

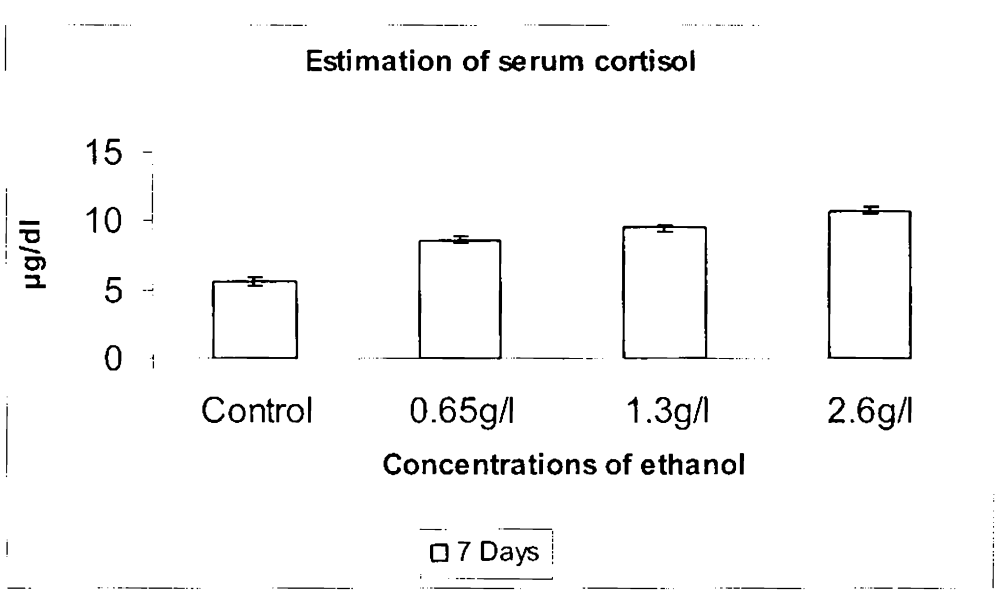
Table 5.2.3a ANOVA Table for serum ferritin

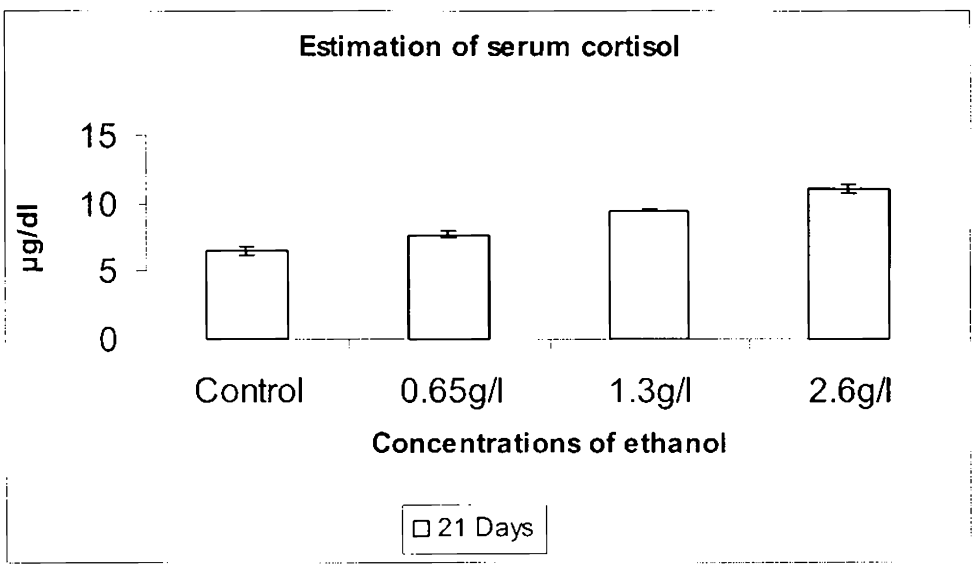
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.060	1	0.060	77.485	0.000
Between Concentrations	0.216	3	0.072	93.806	0.000
Days of Exposure × Concentration	0.084	3	0.028	36.429	0.000
Error	0.031	40	0.001		
Total	0.391	47			

df-degrees of freedom

By using Two Factor ANOVA it was noted that serum ferritin levels varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 5.2.4 Levels of cortisol in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





From the graph (Figure 5.2.4) it can be concluded that serum cortisol levels exhibited significant ($P<0.001$) alterations in *O. mossambicus* during immediate and prolonged exposure periods. Employing ANOVA justifies the above statement (Table 5.2.4a).

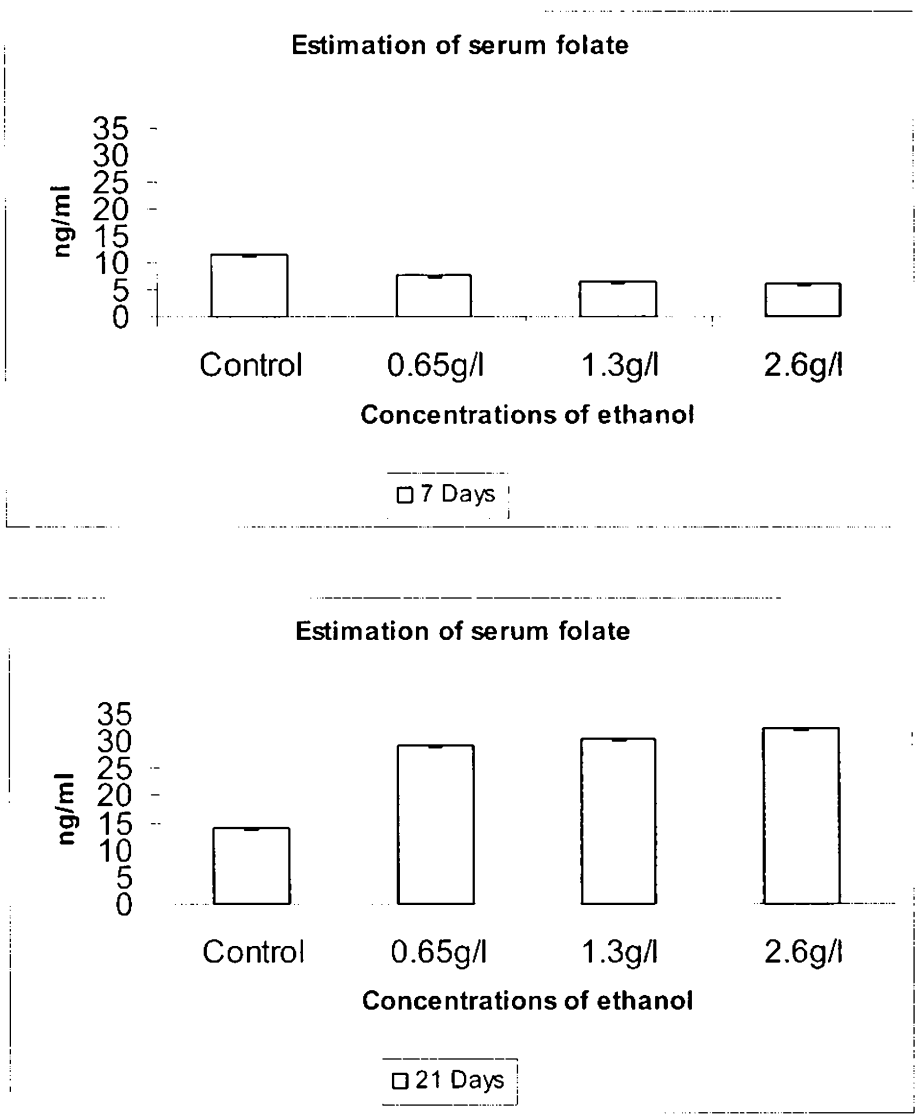
Table 5.2.4a ANOVA Table for serum cortisol

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.064	1	0.064	0.841	0.365
Between Concentrations	154.328	3	51.443	678.047	0.000
Days of Exposure × Concentration	5.310	3	1.770	23.330	0.000
Error	3.035	40	0.076		
Total	162.736	47			

df-degrees of freedom

Statistical analysis done by using Two Factor ANOVA revealed that between days no significant deference was observed for serum cortisol levels. It was noted that between concentrations significant difference ($P<0.001$) was observed. It was concluded that by taking into consideration both the days as well as concentrations (Interaction) significant difference ($P<0.001$) was observed.

Figure 5.2.5 Levels of folate in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Relevant significant ($P<0.001$) changes were noted in the serum folic acid levels (Figure 5.2.5) in *O. mossambicus* exposed to 7 and 21 days of exposure period. Analysis using ANOVA authenticate this (Table 5.2.5a).

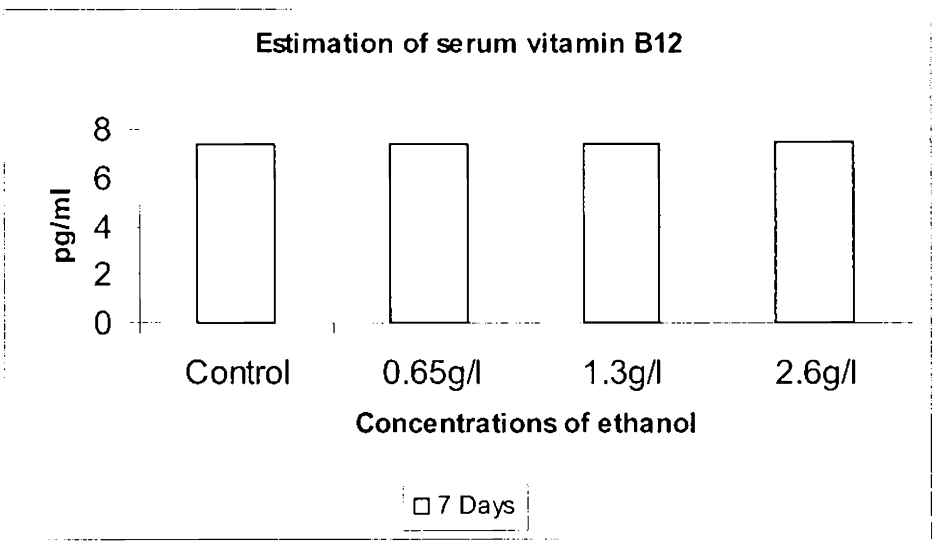
Table 5.2.5a ANOVA Table for Serum Folic Acid

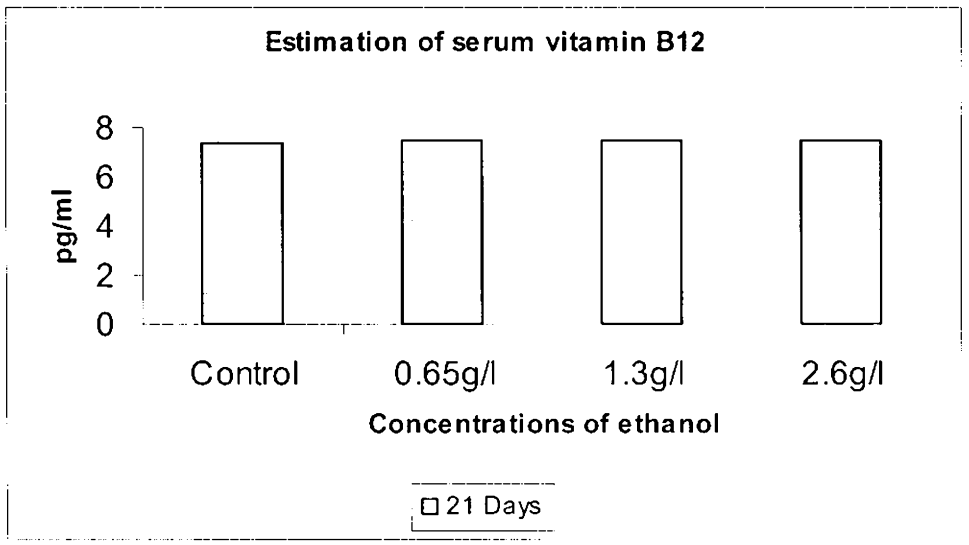
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	4126.819	1	4126.819	85959.723	0.000
Between Concentrations	327.629	3	109.210	2274.789	0.000
Days of Exposure × Concentration	1060.473	3	353.491	7363.052	0.000
Error	1.920	40	0.048		
Total	5516.841	47			

df-degrees of freedom

Studies done by using Two Factor ANOVA indicated that serum folic acid levels varied significantly between days ($P<0.001$). There was a significant difference ($P<0.001$) between concentrations. While comparing both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was noted.

Figure 5.2.6 Levels of vitamin B12 in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Serum vitamin B12 levels were found to be significantly increased ($P<0.001$) (Figure 5.2.6) when *O. mossambicus* was exposed for 7 and 21 days to various sub lethal ethanol concentrations with respect to control. Statistical analysis using ANOVA corroborated the above statement (Table 5.2.6a).

Table 5.2.6a ANOVA Table for serum vitamin B12

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.011	1	0.011	3381.105	0.000
Between Concentrations	0.027	3	0.009	2644.047	0.000
Days of Exposure × Concentration	0.006	3	0.002	606.025	0.000
Error	0.000	40	3.35E-006		
Total	0.044	47			

df-degrees of freedom

Two Factor ANOVA table revealed that serum vitamin B12 levels varied significantly between days ($P<0.001$). Between concentrations there was a significant difference ($P<0.001$). Also while taking into consideration the effects of both the days as well as concentrations (Interaction) significant difference ($P<0.001$) was observed.

Table 5.2.7 Multiple Comparison Test

Subsequent comparisons by multiple comparison tests using Dunnett's is shown below

	Groups	Serum Ferritin	Serum Cortisol	Serum Folic Acid	Serum Vitamin B12
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001 and not significant at b.

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. When being compared with the control all the four serum parameters such as serum cortisol, ferritin, folic acid and vitamin B 12 exhibited significant difference (P<0.001) at all the four sub lethal concentrations.

5.2D DISCUSSION

In the aquatic environment fishes are exposed to numerous adverse impacts that can cause stress reactions in them. The responses to stress typically involve all levels of animal organisation and are collectively called the integrated stress response (Wendelaar Bonga, 1997). Exposure of fish to stressful but sub lethal concentrations of toxic water chemicals would cause acute and/or chronic changes in blood composition (Barton and Iwama, 1991) and stimulate the release of adrenocorticotrophic hormone from the pituitary (Neff, 1985). This in turn, stimulates the synthesis and release of cortisol that is a primary circulating glucocorticoid in teleosts. As released from the adrenal gland into the blood, cortisol will result in hyperglycemia and hypercholesterolemia (Andersen *et al.*, 1991; Grant and Mehrle, 1973; Neff, 1985; Van Raaij *et al.*, 1995). The rise of these energy reserves in response to pollution could be due to the fact that excess energy reserves (as glucose, triglycerides and cholesterol) are required by organisms to mediate the effects of stress (Lee *et al.*, 1983; Shulman, 1974). Stressful stimuli elicit rapid

secretion of both glucocorticoids (Fagerland, 1967; Wedgmeyer, 1969) and catecholamines (Nakano and Tomlinson, 1967) from adrenal tissues of fish. This primary stress hormone in teleost fishes stimulates the synthesis of energy substrates (Vijayan *et al.*, 1997) and thus influences the energy metabolism of fish during stress (Peter *et al.*, 2007; Wendelaar Bonga, 1997). Acute stress conditions activate the teleost hypothalamo-pituitary-adrenal (HPA) axis to release cortisol, the major glucocorticosteroid in fish. Cortisol tends to maintain catecholamine induced hyperglycemia by stimulating protein catabolism and gluconeogenesis, and it promotes lipolysis and has a role in osmoregulation. An increase in the serum cortisol value (Fig 5.2.4) was observed when the fish *O. mossambicus* was subjected to immediate and prolonged exposure to ethanol. The increase in the serum cortisol value obtained during immediate exposure could be attributed to increased metabolic activity brought about by the intake of the toxicant ethanol. The observations made by Baretto and Volpato (2006) on Nile Tilapia when subjected to electroshock and social stressors supports the present finding. In the present study an increase in serum cortisol value obtained during prolonged exposure refers to decreased degradation or excretion of cortisol associated with hepatic or renal derangement caused by chronic exposure of ethanol. The findings cited by Mendelson *et al.* (1966) supports the present data.

Sullivan and Herbert (1964) were among the first investigators to recognize that ethanol has an effect on folic acid status. The term "folate" is a generic descriptor for a family of compounds with chemical structures similar to pteroylmonoglutamic acid, commonly known as folic acid. Folic acid consists of a para-amino benzoic acid (PABA) moiety linked by a methylene bridge to a pteridine ring. The pteridine ring is in turn linked to glutamic acid by a peptide bond. The principal function of folate, especially in its reduced and polyglutamylated form, is that of a coenzyme, accepting and donating one carbon unit in reactions involved in the metabolism of amino acids. A significant decrease ($P<0.001$) (Fig 5.2.5) in serum folic acid observed when subjected to ethanol exposure for 7 days is probably due to folic acid malabsorption. Praneet *et al.* (2003) stated that identical changes have been found in the serum of tobacco smokers. Observations made by Forman (1988) supports the present finding and he too observed similar decrease in serum

folate in acute toxicity studies using ethanol. In the case of prolonged exposure an increase in serum folic acid (Fig 5.2.5) was found which was mainly due to the altered hepatic function of the liver since liver is the major site of folic acid storage and metabolism. This resulted in impaired enterohepatic recycling of folic acid. Similar findings have been made by Carreras *et al.* (2003) who too observed an increase in serum folic acid in rats when subjected for 21 days with ethanol.

A significant increase ($P<0.001$) (Fig 5.2.6) in the serum vitamin B12 was observed when *O. mossambicus* was subjected to immediate and prolonged exposure to ethanol. The increase in the serum Vitamin B12 value was due to the release of vitamin B12 from the ethanol-damaged liver. Our results indicates that a positive relationship exists between serum Vitamin B12 and hepatic enzyme serum levels. A possible explanation for this phenomenon may be the failure of the damaged liver to take up cobalamin and its analogue from the serum. Another possible explanation may lie in the finding that during hepatic damage, vitamin B12 binding and storage of transcobalamin in the liver tissue is disrupted and this results in leakage of vitamin B12 from the liver into the circulation. Observations made by Baker *et al.* (1998) support the present finding.

Ferritin is an iron storage protein, present mainly in the cells of the liver and reticulo endothelial system. The serum concentration of this protein usually reflects the magnitude of the iron stores in the body (Lipschitz *et al.*, 1974). In the present study an increase in serum ferritin level was found when the fish *O. mossambicus* was subjected to 7 days of exposure to ethanol. This was mainly due to the injury caused to liver which resulted in the elevation of serum ferritin level (Prieto *et al.*, 1975). In the case of prolonged exposure a significant increase in serum ferritin level was found. This was mainly due to the severity of liver damage which resulted in the elevation of serum ferritin level. The findings cited by Matti Valimaki *et al.* (1983) supports this. They observed increase in serum ferritin levels in chronic alcoholics. Increase in serum ferritin on prolonged exposure was in turn supported by Baraona *et al.* (1977).

CONCLUSION

Ethanol should be listed under toxic pollutants to fish where it may bring about toxic manifestations in gills and cause liver dysfunction. Therefore, for public health concern, the industrial drainage water should be treated before entering the water resources by removal of chemical pollutants through evaporation, distillation, precipitation and ionic exchange units. Serum biochemistry could be used as a sensitive tool to assess the aquatic impact in contaminated ecosystems and also would be beneficial in determining the baseline health and physiology of aquatic organisms.

Chapter 6

EFFECT OF ETHANOL ON RBC MEMBRANE STABILITY

Contents

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6.1C Results

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6.1A Introduction

An inherent property of cells is their ability to maintain the structural and functional integrity of their membranes under changing environmental conditions. Simple components of biological systems, such as cells, biomolecules and artificial membranes were used in *in vitro* studies since they allow the reduction of the biological variables and more precisely helps in defining and controlling the exposure parameters, compared with *in vivo* exposure. Fish erythrocytes were used to study adaptive responses to ethanol induced changes at the membrane level within short time spans as these cells are nucleated and express many functions as that of somatic cells. Unlike anucleated mammalian RBCs the nucleated RBCs of lower vertebrates preserve both nucleus and mitochondria and can provide an attractive "stripped down" model to study the effect of organic pollutants on cellular compartments (Dey *et al.*, 1993). *In vitro* studies would contribute to clarify the fundamental mechanisms of biological effects brought about by ethanol induced toxicity. By using *In vitro* studies, it was shown that ethanol brings about chaotropic effects on membrane and cytoskeleton proteins denaturation. It is a well known

denaturating agent that can promote the exposure of polar groups in the protein unfolding processes. For this reason it has been stated that, incorporation of ethanol to the membrane environment is associated with the promotion of exposure of polar groups resulting in a membrane denaturation. Furthermore, there is evidence stating that alcohol causes membrane deformity, and brings about modification in the osmotic fragility of different cell types (Sozmen *et al.*, 1994). Freshly drawn blood samples mixed with an anticoagulant were used for erythrocyte membrane analysis. Erythrocyte membrane is often used as a model membrane in investigating the structure and the functions of the biological membranes as well as in studying the influence of different physical and chemical factors on the membranes. Incubation of normal erythrocytes with ethanol facilitates hemolysis and increases the percentage of cells that were hemolysed at maximal rate. There is an especially great need for studies on the impact of these compounds on the properties of blood and other tissues which results in their distribution to other sites in the body. The present study describes the damaging effects of ethanol and their metabolites on the erythrocyte membrane. Cunha *et al.* (2007) has stated that erythrocytes suffer lysis depending upon the concentrations of ethanol. Previous reports are not available on the effect of ethanol on erythrocyte membrane of the fresh water teleost, *O. mossambicus*. Therefore this study was designed to investigate the effect of ethanol on the stability of erythrocyte membrane. The direct study of ethanol on membrane *in vitro* can show the basic aspects of its effects on cellular metabolism. This study was designed to investigate the alterations in the membrane stability after *in vitro* exposure of fish erythrocytes to different concentrations of ethanol.

6.1B Materials and Methods

Collection, maintenance, acclimatization of fish and determination of LC_{50}, was the same as that described in chapter 1B, Section 1.2B.1 to 1.2B.5.

6.1B1 *In-vitro* studies.

For conducting RBC membrane stability studies in *In-vitro* conditions, *O. mossambicus* of 10 ± 2 g were taken. Fishes were sacrificed by a blow to the head and blood was drawn from the common cardinal vein using 1ml sterile plastic

insulin syringe (Smith *et al.*, 1952) (26mm gauge size) containing sodium citrate as an anticoagulant (5mg/ml) (1:5 dilution) (Oser, 1976).

6.1B1.1 Isolation of red blood cells

Erythrocytes were used within one hour of collection, and with each experiment lasting no longer than 4 hour. Erythrocytes were isolated by centrifuging at 3000 rpm for 5 minutes. After removal of plasma and buffy coat, the red blood cells were further washed three times with three volumes in the same isotonic medium and were concentrated by centrifugation under the same conditions. In order to study the effect of ethanol on erythrocyte membrane, 0.5 ml of ethanol was directly added in the tubes in which the final ethanol concentrations in the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively. Hemolysis was determined by measuring the absorbance of hemoglobin in the supernatant at 540 nm. Hemolysis was expressed by measuring the hemoglobin concentration. Erythrocyte membrane was often used as a model membrane in investigating the structure and the functions of the biological membranes as well as in studying the influence of ethanol on the membranes.

6.1B2 Estimation of RBC membrane stability (*In vitro* condition)

Reagents Needed

1. Tri sodium citrate (500 mg%)
2. 154 mM Isotonic NaCl in 10 mM Sodium phosphate buffer, pH 7.4
3. 85.47 mM Hypotonic NaCl in 10 mM Sodium phosphate buffer, pH 7.4

Procedure

Stock RBC suspensions were prepared after washing the cells thrice with isotonic saline. Different volumes of the suspension were mixed with distilled water of known volume to hemolyse the cells. It was then centrifuged at 1000g for 5 minutes. The absorbance of the supernatant was read at 540 nm against distilled water as blank. The dilution giving a suitable absorbance for 100% hemolysis was selected. Also a suitable volume of blood giving an absorbance for 100% hemolysis was noted.

The experiment was carried out with each of the three concentrations of ethanol as described below.

1. To 0.1 ml of the stock RBC suspension in a centrifuge tube, 5 ml of isotonic saline was added and incubated for 30 minutes at room temperature. It was then centrifuged at 1000g for 5 minutes. The absorbance of the supernatant was read at 540 nm. This gave the absorbance of the "blank" (B).
2. To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of distilled water was added and incubated for 30 minutes at room temperature. To this 0.5 ml of ethanol stock solution was added (in which the final concentration of ethanol in each of the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively). It was then centrifuged at 1000g for 5 minutes and the absorbance of the supernatant was read at 540nm. This gave the absorbance corresponding to 100% hemolysis (H).
3. To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of hypotonic saline was added and incubated for 30 minutes at room temperature. To this 0.5 ml of the ethanol stock solution was added (in which the final concentration of ethanol in each of the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively). It was then centrifuged at 1000g for 5 minutes and the absorbance of the supernatant was read at 540 nm. This gave the absorbance of the control (C).
4. To 0.1 ml of the stock RBC suspension in three centrifuge tubes, 4.5 ml of hypotonic saline followed by 0.5 ml of the ethanol stock solution(such that the final concentration of ethanol in each of the tubes were 0.65 g/l, 1.3 g/l and 2.6 g/l respectively) was added. It was then incubated for 30 minutes at room temperature. It was then centrifuged at 1000g for 5 minutes and the absorbance of the supernatant obtained was read at 540 nm. This gave the absorbance corresponding to the "test" (T).

6.1C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l of ethanol on erythrocyte membrane stability in *O. mossambicus* was given in Table 6.1.1 to 6.1.4 and in Figure 6.1.2.

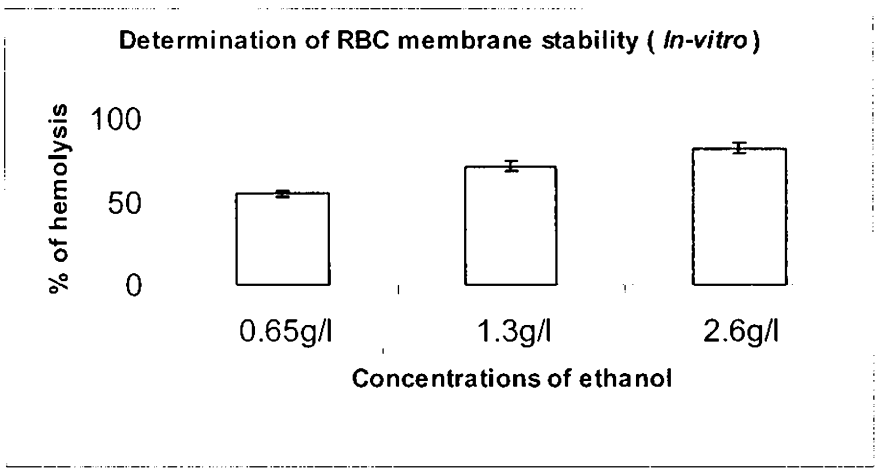
The results obtained were analyzed statistically using One-Way ANOVA of the raw data, followed by Dunnett's method. *In-vitro* studies conducted on the RBC membrane stability revealed that ethanol had a labilising effect on the erythrocyte membrane. 2.6 g/l exhibited maximum percentage of hemolysis (82.51%) followed by 1.3 g/l and 0.65 g/l which gave percentage hemolysis of (71.29%) and (54.84%) respectively.

Table 6.1.1 Percentage hemolysis in *O. mossambicus* on exposure to different concentrations of ethanol (*In - vitro* conditions)

Groups	% Hemolysis
Control	0
0.65g/l	54.84 ± 1.9265
1.3g/l	71.29 ± 3.1025
2.6g/l	82.51 ± 2.4627

Average of six values in each group ± SD of six observations

Figure 6.1.2 Percentage hemolysis in *O. mossambicus* on exposure to different concentrations of ethanol (*In - vitro* conditions)



RBC membrane exhibited an increase in hemolysis (Table 6.1.1 and Figure 6.1.2) in *in-vitro* conditions, and was found to be depended upon dosage. This was confirmed by using ANOVA (Table 6.1.3) and the result is depicted below.

Table 6.1.2a ANOVA Table for RBC (*In-vitro*)

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	2324.344	2	1162.172	179.702	0.000
Error	97.008	15	6.467		
Total	2421.352	17			

df - degrees of freedom

Statistical analysis by using One Way- ANOVA revealed that there was an overall significant change (P<0.001) between concentrations in the RBC membrane stability levels during *in-vitro* conditions.

6.1.3 Multiple Comparison Test

A subsequent comparison between different concentrations of ethanol was done by Dunnett's method and the results were depicted in the Table 6.1.3

	Groups	RBC (*In-vitro*)
Dunnett	0.65g/l Vs 1.3g/l	0.000[a]
	0.65g/l Vs 2.6g/l	0.000[a]

The values are significant at a = P < 0.001.

In the case of RBC membrane stability studies in *in-vitro* conditions significant difference (P<0.001) was observed at 1.3 g/l and 2.6 g/l when being compared with 0.65g/l.

6.1D Discussion

The result from this experiment indicated that erythrocyte membrane was maximally damaged and has spilled its haemoglobin content depending upon the concentration of ethanol. The levels of released hemoglobin serves as an indicator of hemolysis, caused by increased membrane fragility. Fragility is referred to as a

membrane associated phenomenon which reflects the susceptibility of red cells to lysis. This in turn explains that RBC membrane has become more fragile when treated with ethanol. Also the change in cation concentration brings about increased fragility. This results in the increased leakage of cations from the cells resulting in swelling of the cell, thus ultimately leading to lysis. Niranjan and Krishnakantha. (2000) observed similar increase in the osmotic fragility of erythrocytes in rats fed with oxidized ghee, supports the present finding. The present data confirm that, ethanol brings about an increased disintegration of erythrocytes. An increase in the RBC hemolysis was observed when *O. mossambicus* was subjected to different concentrations of ethanol in *in-vitro* conditions. In the present study as the concentration of ethanol increased hemolysis of RBCs also increased (Table 6.1.1 and Figure 6.1.2). Chi and Wu (1991) observed similar state of increased rate of hemolysis of red blood cells when mediated by ethanol. The hemolysis rate increased depending upon the increased concentration of ethanol. Changes in the ion transport and in the osmotic fragility are considered as indicators of alterations in the erythrocyte membrane (Baranski *et al.*, 1974; Kovacs *et al.*, 1997). Similar observations in hemolysis were made by Cleary *et al.* (1982) on rabbit erythrocytes subjected to micro waves in *in-vitro* condition. Another reason for the labilising effect is the membrane damage brought about by the direct effect of lipid peroxidation products. The observations cited by Das and Vasudevan (2005) supported the present finding. Erythrocytes are prone to oxidative damage due to presence of polyunsaturated fatty acids, heme, iron and oxygen (Kameda *et al.*, 1985). Red blood cell membrane being rich in polyunsaturated fatty acids are very susceptible to free radical mediated peroxidation. Lipid peroxidation mediated by free oxygen radicals, is believed to be an important cause of destruction and damage to cell membranes, since polyunsaturated fatty acids of the cellular membranes are degraded by this process with consequent disruption of membrane integrity. From these results it can be concluded that RBC hemolysis and lipid peroxidation by ethanol can be one of the molecular mechanisms involved in ethanol induced toxicity studies (Armutcu *et.al.*, 2005). Thus, the elevated lipid peroxide concentration in the erythrocytes probably reflects a higher production of peroxyl radicals, which ultimately leads to the peroxidation of PUFA in these cells. This in

turn leads to increased fragility of the cell membranes which ultimately brings about lysis of the cells (Muduuli *et al.*, 1982). The present investigation points out that ethanol brings about maximum damage to the RBCs when subjected to different concentrations of ethanol in *in-vitro* conditions.

6.2 *IN-VIVO* RBC MEMBRANE STABILITY STUDIES

Contents

6.2A Introduction
6.2B Materials and Methods
6.2B.1 Estimaiton of RBC membrane stability (*In-vivo* Condition)
6.2C Results
6.2D Discussion

6.2A Introduction

Red blood cells possess a much simple structure. It is composed of a single membrane surrounding a solution of haemoglobin (this protein forms about 95% of the intracellular protein of the red cell) (Robert *et al.*, 2000). The erythrocyte membrane has long been served as a convenient model system employed for studying the chemical and physical properties of cell membrane due to its relative simplicity (Salil and Shyamali, 1999). Lipids are crucial structural components of biomembranes which dictate the integrity of the membranes (Hummel, 1993). Membrane lipid composition directly reflects the membrane properties (Yeagle, 1985). Cholesterol, one of the major components of the membranes, plays a central role in membrane biosynthesis, integrity and cell growth. It also regulates membrane fluidity. The functions of membrane are therefore determined by membrane composition and organization (Levin *et al.*, 1990). Erythrocytes (RBCs) are frequently used to evaluate oxidative stress. The RBC membrane is rich in polyunsaturated fatty acids, a primary target for reactions involving free radicals, which seems to be very susceptible to lipid peroxidation (Devasena *et al.*, 2001). Lipid peroxidation is an autocatalytic process, which ultimately results in cell death (Dsouza and Dsouza 2002). An oxidative cellular defect in the red blood cell (RBC) can accentuate oxygen radical formation and risk damage to cellular components (Flynn *et al.*, 1985; Scott 1993). Ethanol disrupts the physical structure of cell membranes (Goldstein, 1986), and thus brings about oxidative damage. Oxidative damage alters membrane permeability and eventually lead to hemolysis (Lubin and

Chiu, 1992). Erythrocytes are exposed to continuous oxidative stress due to continuous generation of free radicals brought about by the oxidation of haemoglobin. In addition, oxidative damage causes immune recognition of RBC (Low *et al.*, 1985).

The most commonly used method of erythrocyte ghost preparation is to hemolyse RBC in hypotonic solutions to remove hemoglobin. Even though this technique is widely used, certain important variables, namely pH and ionic strength of the hemolyzing solutions, appear partially responsible for conflicting reports on the composition and function of RBC membrane (Stubbs and smith, 1984). While these studies have identified oxidative damage as a major determinant of RBC survival, the detailed mechanism of these damages to RBC brought about by ethanol remains largely ill-defined. Membrane lipids are susceptible to peroxidation induced damage as they are largely composed of polyunsaturated fatty acids. Peroxidative reactions involving free radicals in lipid domains results in damage to integral membrane proteins, leading to alteration of membrane dynamics and function (Sevanian and Hochstein, 1985). The accumulation of activated oxygen causes hemolysis (Hebbel, 1986; Shinar and Rachmilewltz, 1990 and Saltman, 1989). However, the chemical composition of the red blood cell (RBC) membrane may vary depending on the methods used to isolate the membrane (Dodge *et al.*, 1963; Ponder, 1961 and Weed and Lacelle, 1969). For the present study, fish red blood cells (RBCs) has been used as a model system to delineate the effects of oxidative damage brought about by ethanol on red blood cells of *O. mossambicus*. The cellular membrane is a lipid bilayer essentially constituted by phospholipids, cholesterol and glycolipids. Small variations in percentage composition and molar ratio of the different classes of phospholipids and glycolipids, as modifications in the composition in fatty acid and cholesterol amount, result in changes of the physical-chemical status (with implications on membrane's fluidity and permeability) of enzyme's activity and/or of channels and ionic pumps constituted by intrinsic membrane proteins. Moreover, in these molecules, a difference in composition in the fatty acids can result in a greater sensibility to peroxidative stress, with a consequent increase in membrane fragility (Angela *et al.*, 2007).

6.2 B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design was the same as that described in chapter 1, section 1.2B.1 to 1.2B.5. Isolation of red blood cells remains the same as described in 6.1.B1.1.

6.2B.1 Estimation of RBC membrane stability (*In vivo* condition)

Reagents Needed

1. Tri sodium citrate – 500 mg%
2. 154 mM isotonic NaCl in 10 mM sodium phosphate buffer, pH 7.4
3. 85.47 mM hypotonic NaCl in 10 mM sodium phosphate buffer, pH 7.4

Procedure

Blood containing citrate as an anticoagulant was employed for membrane stability studies. It was then centrifuged at 4^0C in a refrigerated centrifuge at 3000 rpm for 30 minutes. The pellet obtained contains erythrocytes. It was then washed thrice with isotonic saline solution. Different volumes of erythrocyte suspensions were prepared by mixing with distilled water. This hemolyses the cells which were then centrifuged at 1000g for 5 minutes. The supernatant obtained was taken. It was then read at 540 nm against distilled water as blank. The RBC suspension which gives a suitable absorbance for 100% hemolysis was selected and this was used as stock. Also a suitable volume of blood giving a suitable absorbance for 100% hemolysis was noted.

To 0.1ml of the stock RBC suspension, 5 ml of isotonic saline was added. It was then incubated for 30 min at room temperature. After incubation it was centrifuged at 1000g for 5 min. The supernatant which contains haemoglobin was read at 540 nm. This serves as the 'blank' (B). Similarly 5 ml of distilled water was added to 0.1 ml of the stock RBC suspension taken in a centrifuge tube. It was then incubated for 30 min at room temperature. After incubation it was centrifuged at 1000g for 5 min and the absorbance of the clear supernatant was measured at

540 nm. This gave the absorbance corresponding to 100% hemolysis (H). In the third set, 4.5 ml of hypotonic saline and 0.5ml of distilled water were added to 0.1 ml of the stock RBC suspension in a centrifuge tube. It was then incubated for 30 min at room temperature. After incubation the tubes were centrifuged at 1000g for 5 min and the absorbance of the supernatant was read at 540nm. This gave the absorbance corresponding to the control(C). To the fourth set, 4.5 ml of hypotonic saline was added to 0.1 ml of stock RBC suspension. It was then incubated for 30 min at room temperature. After incubation 0.5 ml of distilled water was added and it was then centrifuged at 1000g for 5 min. The absorbance of the supernatant was read at 540nm. This gave the absorbance corresponding to the test (T).

6.2C Results

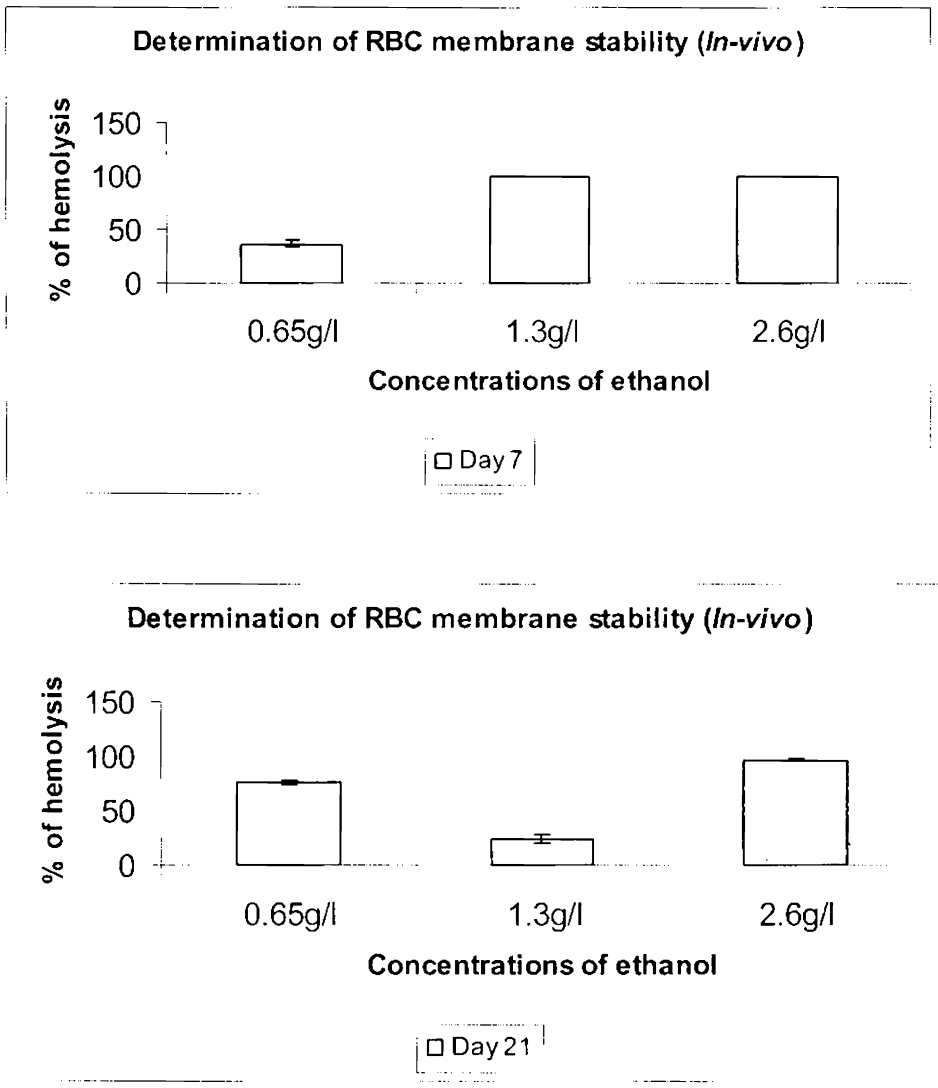
Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l of ethanol on RBC membrane stability in *O. mossambicus* are given in Table 6.2.1 to 6.2.3 and Figure 6.2.2. The results obtained on exposure to the three sub lethal concentrations of ethanol for 21 days followed by a periodical sampling at 7 days were analyzed statistically using Two Way ANOVA of the raw data, followed by Dunnett's method. It exhibited that as the concentration of ethanol increases, the labilisation of erythrocyte membrane also increases. Exposure to 2.6 g/l concentration of ethanol exhibited highest labilisation effect on *O. mossambicus* when being subjected for 7 days and 21 days experiment. Minimum effect of labilisation was seen in 0.65 g/l in the case of 7 days of exposure whereas on prolonged exposure, minimum effect was exhibited by 1.3 g/l.

Table 6.2.1 Percentage hemolysis in *O. mossambicus* on exposure to different concentrations of ethanol for 7 days and 21 days (*In- vivo* conditions).

Days of Exposure	Groups		
	0.65g/l	1.3g/l	2.6g/l
7 days	36.60± 2.8958	99.55± 0.0848	99.92± 0.0799
21 days	76.59± 1.6613	24.75± 3.8241	96.60± 0.6665

Average of six values in each groups ± SD of six observations.
Values are expressed in %

Figure 6.2.2 Levels of RBC Membrane stability (*In-Vivo*) in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



A significant difference ($P<0.001$) in RBC membrane stability was observed in *O. mossambicus* when treated with various sub lethal concentrations of ethanol with respect to control during 7 and 21 days of exposure period (Table 6.2.1 and Figure 6.2.2). This was statistically supported by employing ANOVA and the results obtained is shown below (Table 6.2.2a).

Table 6.2.2a ANOVA Table for RBC (*In-vivo*)

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	1453.389	1	1453.389	332.489	0.000
Between Concentrations	12284.809	2	6142.404	1405.186	0.000
Days of Exposure × Concentration	20161.551	2	10080.776	2306.159	0.000
Error	131.137	30	4.371		
Total	34030.886	35			

df - degrees of freedom

Statistical analysis done by using Two Factor ANOVA indicated that RBC membrane stability levels varied significantly between days ($P<0.001$). There was a significant difference ($P<0.001$) between concentrations. While comparing both the days as well as concentrations (Interaction) effect together, significant difference ($P<0.001$) was noted.

Table 6.2.3 Multiple Comparison Test

	Groups	RBC (*In-vivo*)
Dunnett	0.65g/l Vs 1.3g/l	0.000[a]
	0.65g/l Vs 2.6g/l	0.000[a]

The values are significant at a = $P < 0.001$.

6.2D Discussion

The ability of organic solvent such as ethanol to destabilize the membrane as well as the membrane proteins correlates to their cytotoxicity. The present experimental data indicated that erythrocyte membrane was maximally damaged and exhibited increased rate of hemolysis when treated with ethanol. This is due to the direct effect of lipid peroxidation products. Membrane peroxidation lead to changes in membrane fluidity, permeability and also results in the enhanced rates of protein

degradation, which will eventually lead to cell lysis. The deformity of destructed RBC and increased rate of RBC hemolysis results in the increased production of free radicals. High levels of polyunsaturated fatty acids (PUFA), continual exposure to high concentrations of oxygen or the presence of iron, a powerful transition-metal catalyst, render erythrocytes highly susceptible to peroxidative damage (Clemens and Waller, 1987). Also ethanol exhibits a denaturating effect on erythrocyte membrane which is also been associated with abnormal RBC morphology resulting in an increased susceptibility to hemolysis (Prokopieva *et al.*, 2000; Chi *et al.*, 1990).

In the present study an increase in RBC hemolysis (Figure 6.2.2) was observed when *O. mossambicus* was exposed to ethanol for 7 days. This increase in RBC hemolysis is due to the increased activities of serum specific enzymes. Ivanov (2001) observed increase in RBC destruction and hemolysis when treated with ethanol, supports the present finding. Another reason for the increase in RBC hemolysis is due to membrane lipid peroxidation. RBC membranes are prone to peroxidative damage because they are rich in unsaturated fatty acids and are exposed to high oxygen concentration in the blood. The findings by Niki *et al.* (1988) and Hayam *et al.* (1993) supports the above observation. Xenobiotics are oxidized to free radicals within RBCs and induce hemolysis of the RBC membrane (Armutcu *et al.*, 2005), which results in the release of hemoglobin thereby inducing a multitude of toxic effects. The findings of Everse and Hsia (1997) supports the present study. Ivanov (2001) observed similar destruction of RBCs and hemolysis on the membrane when treated with organic solvents.

RBC membranes are prone to peroxidative damage because they are rich in unsaturated fatty acids and are exposed to high oxygen concentration in the blood (Hayam *et al.*, 1993). Membrane lipid composition determines membrane fluidity. Any alteration in the concentration of cholesterol is known to affect the transport functions and activity of membrane enzyme (Stubbs, 1983). It has been suggested that cholesterol regulates the lipid mobility in the membrane in physiological situations (Chailley *et al.*, 1981). The decrease in hemolysis observed at 1.3g/l when *O. mossambicus* was exposed for 21 days indicates that RBC membrane has become rigid. This can be due to the slight increase in the cholesterol/phospholipid ratio. The

observations made by Yeagle *et al.* (1990) and Kuypers *et al.* (1996) supports the above finding. Another possible reason for rigidity can be due to the decrease in unsaturated fatty acids in the erythrocyte membrane followed by an increase in saturated fatty acids, as the saturated fatty acid residues, because of their linear hydrocarbon residues and hydrophobicity, interact very strongly with each other. Oxidative damage has been shown to change a number of RBC properties. A fall in PUFA followed by an elevation of cholesterol increases the rigidity of the phospholipid bilayer. The findings made by Dobrestov *et al.* (1977) supports the present observation. Lipid peroxidation is known to cause polymerization of membrane components, thus decreasing cell deformability (Pfafferott *et al.*, 1962). Bourel *et al.* (1987) suggested that alcohol, or one of its metabolites, induces erythrocyte morphological alterations that correlates with some alterations in the lipid content of the erythrocyte membrane (such as increased cholesterol/ phospholipid ratio). Increased membrane rigidity and decreased RBC deformability can also be induced by oxidative cross-linking of membrane protein.

Beauge *et al.* (1994) observed that alcohol induces rigidification of red blood cell membrane, supports the above observation. Levin *et al.* (1990) have proposed that the oxidation of membrane lipids results in the formation of peroxidation degradation products (such as for e.g. Malondialdehyde, MDA) which leads to the cross linking reactions of the lipid-lipid and lipid-protein type thereby making the membrane more rigid and hence less fluid. Similarly the increase in hemolysis observed at 0.65 g/l and 2.6 g/l on prolonged exposure can be due to the decrease in Hb and PCV values which arises due to the increased fragility of the erythrocytes. Observations made by Patra *et al.* (2001) supports this finding.

It can be concluded that ethanol brings about maximum damage to the RBCs when subjected to different concentrations of ethanol in *in-vivo* conditions.

Chapter 7

EFFECT OF ETHANOL ON METABOLIC PROFILES

Contents

7.1 EFFECT OF ETHANOL ON CARBOHYDRATE METABOLISM OF FRESH WATER TELEOST, *OREOCHROMIS MOSSAMBICUS* (PETERS)

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a. Estimation of Total Carbohydrate
b. Estimation of Blood Glucose
c. Estimation of Serum Lactate Dehydrogenase

7.1C Results

7.1D Discussion

7.1 A Introduction

Pollution has been referred to as an unwelcome gift of rapid industrial revolution and excessive exploitation of natural resources (Jamal *et al.*, 2004). Majority of the industries are water based and untreated or inadequately treated waste water discharged from them into the nearest water body causes water pollution (Pandey and carney, 1998). Water pollution has been recognized globally as a potential threat to both human and other animal populations which interact with the aquatic environments (Biney *et al.*, 1987; Svensson *et al.*, 1995). The pollution of freshwater is increasing at an alarming rate due to steady increase in industrialization and urbanization. Disposal of industrial waste, human waste, including sewage in fresh water bodies causes serious health hazards (Dilshada *et al.*, 2007). Therefore, measuring the biological effect of pollutants seems to be essential for assessing the quality of the aquatic environment (Vijayavel *et al.*, 2006). Within this context, fish has been widely used in toxicologic pathology as

models to evaluate the health of aquatic ecosystems (Law, 2003). Toxicological studies have shown that the concentration of pollutants can change the enzyme activities and often directly induce cell damage in specific organs (Yang and Chen, 2003). In environmental toxicology studies, the application of non-mammalian systems is rapidly expanding, and, for aquatic systems, fish has become an indispensable model system for evaluating the effects of noxious compounds (Blair *et al.*, 1990). According to Neff (1985), changes in certain biochemical parameters in fish blood can reflect acute or chronic pollutant - induced damage. Blood chemistry has long been employed as a helpful diagnostic tool in pathological, toxicological and general clinical tests. Several specific enzymes have been proposed as biomarkers in water pollution (Agradi *et al.*, 2000). Among specific enzymes employed as biomarkers to determine the pollution levels, lactate dehydrogenase occupies a predominant role. The cytoplasmic enzyme LDH is widely used as marker of organ or tissue lesions in toxicology and in clinical chemistry (Das *et al.*, 2004a). Energy metabolism plays a key role as the animal is forced to expend more energy to overcome toxic stress (Begum and Vijayaraghavan, 1999). The main pathway of carbohydrate catabolism in invertebrates is glycolysis which results in the transformation of glucose into pyruvic acid. Fish serum reflects status of many biochemical processes in the metabolism. Therefore, it was emphasized that measurement of serum biochemical parameters can be used as a diagnostic tool in fish toxicology to identify the target organs affected by toxicants and to assess their general health status (McDonald and Grosell, 2006; Zikic *et al.*, 2001). Even though several reports have been cited to assess the effect of industrial effluents on carbohydrate metabolism of fishes mainly on *O. mossambicus*, the present study seems to be a pioneer work assessing the impact of ethanol on carbohydrate metabolism of fresh water fish, *O. mossambicus*.

7.1 B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, Section 1.2B.1 to 1.2B.5.

7.1B.1 Preparation of blood samples for experimental studies

The method remains the same as described in chapter 1, section 1.2B.6.

7.1B.1 Preparation of serum samples for experimental studies

The method remains the same as described in chapter 5, section 5.1B.1.

7.1B.2 Preparation of tissue samples for experimental studies

Tissues such as gill, liver, muscle, kidney and heart were used for carrying out the biochemical analysis. 10% homogenate of gill and muscle tissue, 2% homogenate of liver and 0.5% homogenate of kidney and heart tissue each were prepared in 10% TCA. The homogenates so obtained were centrifuged at 1000g for 15 minutes. The supernatant was taken. They were stored frozen at-20^0C until assayed and thawed immediately prior to analysis.

7.1B.2 Experimental procedures

The following parameters were studied under carbohydrate metabolism

a. Estimation of total carbohydrates

Principle

The total carbohydrate was estimated by the method of Carrol *et al.* (1956) with slight modifications. In this method carbohydrates were first hydrolyzed into simple sugars using acid. In the acidic medium this simple sugar gets dehydrated to hydroxymethyl furfural which then forms a green coloured product with anthrone giving an absorption maximum at 620nm.

Reagents

1. 10% Trichloro acetic acid (TCA)
2. Anthrone reagent: Dissolved 200 mg of anthrone in 100 ml of ice cold 95% Conc. H_2SO_4. This reagent was freshly prepared before use.

Procedure

To 0.2 ml of the supernatant (refer section 7.1B.2), 5 ml of anthrone reagent was added. The tubes were heated to boiling condition for 15 minutes. They were then cooled and the absorbance was read at 620 nm in a spectrophotometer using reagent as blank of the same proportion. The values were expressed as mg of glucose/g wet wt of tissue.

b. Estimation of blood glucose

Principle

Blood glucose was estimated by the method of Sasaki *et al.* (1972) using O-toluidine. Ortho toluidine reacts with glucose in hot acetic acid solution to produce blue-green colour which was measured at 630 nm.

Reagents

1. Ortho toluidine boric acid reagent: This reagent consists of 2.5 g of thiourea and 2.4 g of boric acid in 100 ml solvent, consisting of mixture of water, acetic acid (AR) and ortho toluidine (distilled) in the ratio of 10:75:15.
2. Standard glucose: 100 mg of glucose was dissolved in 0.1% benzoic acid. 10 ml of the above solution was diluted to 100 ml to give 100 µg of glucose per ml.

Procedure

To 0.2 ml of blood, 0.8 ml of 10%TCA was added. The contents were mixed well. The tubes were centrifuged at 1000g for 5 minutes. 0.5 ml of the supernatant was taken. To this 2.0 ml of ortho toluidine reagent was added. The tubes were then heated in a boiling water bath for 15 minutes along with a standard solution containing 20-100 µg of glucose. The blue colour developed was read at 630 nm in a spectrophotometer. The results obtained were expressed as mg/dl.

c. Estimation of serum lactate dehydrogenase (LDH) (L-lactate: NAD Oxidoreductase; E.C 1.1.1.27)

Principle

The serum lactate dehydrogenase was estimated by the method of King *et al.* (1959, 1965b). The lactate obtained was acted upon by lactate dehydogenase to form pyruvate in the presence of NAD^+. The pyruvate forms pyruvate phenyl hydrazone with 2,4 dinitrophenyl hydrazine. The color developed was read in a spectrophotometer at 440 nm.

Reagents

1. Glycine buffer, 0.1 M, pH 10: 7.505 g of glycine and 5.85 g of sodium chloride were dissolved in 1 litre of water.
2. Buffered substrate: 125 ml of glycine buffer and 75 ml of 0.1 N NaOH were added to 4 g of lithium lactate. The contents were mixed well.
3. Nicotinamide adenine dinucleotide: 10 mg of NAD^+ was dissolved in 2.0 ml of water.
4. 2,4 Dinitrophenyl hydrazine: 20 mg of DNPH was dissolved in 100 ml of 1 N HCL.
5. 0.4 N NaOH
6. Standard, 1 µmol/ml: 11 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate (1 µmole of pyruvate/ml).
7. NADH solution, 1µmol/ml: 8.5 mg of NADH solution was dissolved in 10 ml buffered substrate.

Procedure

Placed 1.0 ml of the buffer substrate and 0.1 ml of serum sample into each of the two tubes labeled "Test' and "Control". Simultaneously 0.2 ml of water was added into another test tube labeled as "blank" followed by the addition of 1.0ml of buffer substrate. Then to the test tube labeled as "Test" 0.2 ml of NAD^+ was added.

It was then mixed and incubated at 37^{0}C for 15 min. Exactly after 15 minutes, 1.0 ml of dinitrophenyl hydrazine was added to both the tubes labeled as test and control. They were then left for further 15 min at room temperature. Then to all the tubes 10 ml of 0.4N NaOH was added and the colour developed was read immediately at 440 nm against reagent taken as blank. Simultaneously a standard curve with sodium pyruvate solution of concentration ranging from 0.1-1.0 μ mol was taken. The enzyme activity so obtained was expressed as U/L in serum.

7.1C Results

On exposure to 0.65 g/l, 1.3 g/l and 2.6 g/l ethanol for a period of 7 and 21 days, parameters like serum lactate dehydrogenase, blood glucose and total carbohydrate levels in the tissues of *O. mossambicus* exhibited significant variations from that of control. The results of 7 and 21 days exposure to ethanol are depicted in table 7.1.1 to 7.1.7 and in figures 7.1.3 to 7.1.5. Results obtained were then statistically analyzed by ANOVA (Analysis of Variance) followed by Tukey's test and Dunnett's method.

Table 7.1.1 Effect of exposure to different concentrations of ethanol for 7 days on carbohydrate metabolism of *O. mossambicus*.

Parameters Investigated	Sample Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Glucose (mg/dl)	Blood	31.50± 1.0488	54.33± 1.2111	94.00± 2.3664	134.3± 3.3267
Lactate Dehydrogenase (U/L)	Serum	8.767± 0.4131	20.78± 1.2465	42.50± 1.8708	80.00± 1.4142
Total Carbohydrate (mg/g wet weight of tissue)	Gills	2.157± 0.0726	1.670± 0.0651	1.397± 0.0602	1.046± 0.0742
	Muscle	4.825± 0.2141	3.390± 0.0447	3.147± 0.0308	2.685± 0.0967
	Liver	51.08± 1.7438	38.52± 1.3942	32.33± 0.7396	30.64± 0.3326
	Heart	48.50± 3.3006	40.51± 2.0880	34.68± 1.2201	29.70± 1.1444
	Kidney	40.66± 2.7945	37.04± 1.6549	34.42± 1.6516	29.21± 1.4678

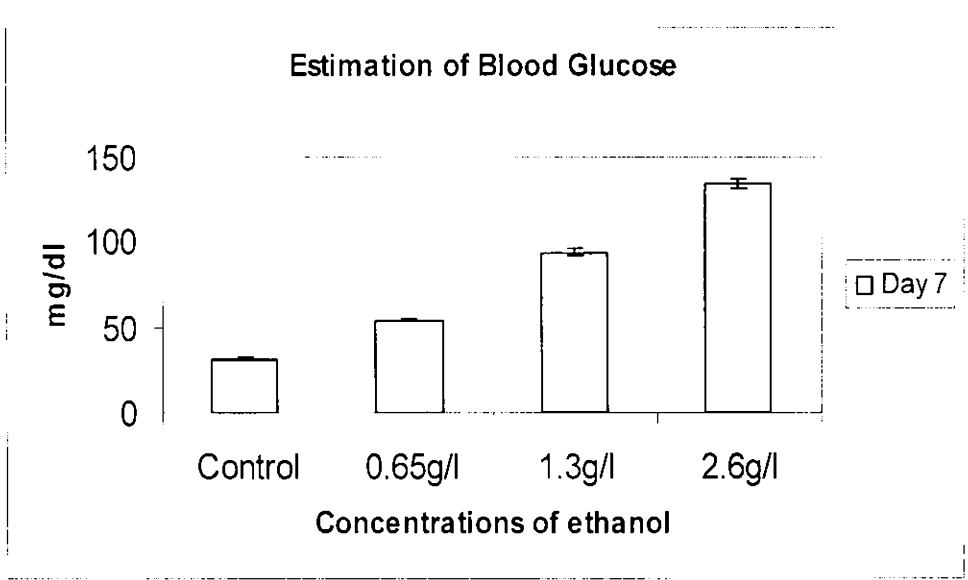
Average of six values ± SD of six observations

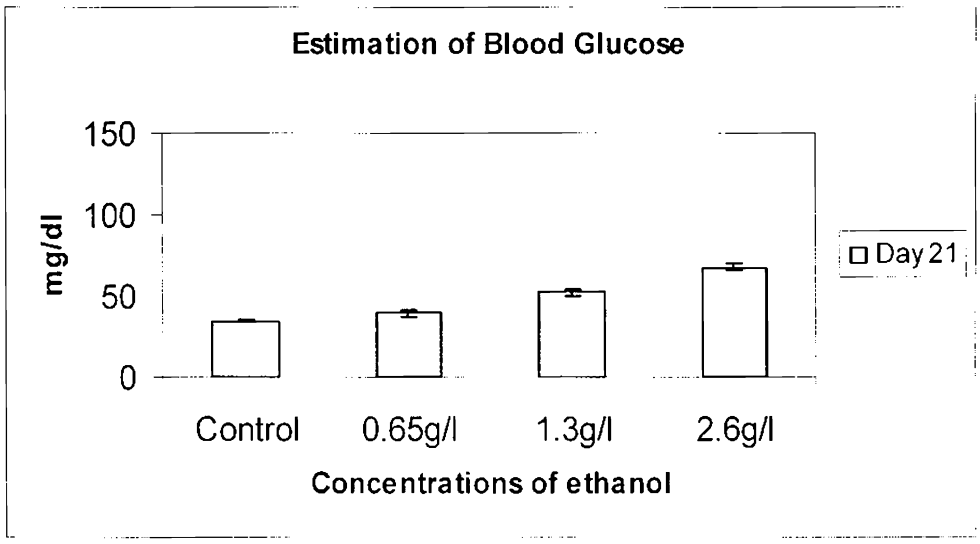
Table 7.1.2 Effect of exposure to different concentrations of ethanol for 21 days on carbohydrate metabolism of *O. mossambicus*.

Parameters Investigated	Sample Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Glucose (mg/dl)	Blood	34.65± 0.9160	39.50± 1.8708	52.50± 1.8708	67.50± 1.8708
Lactate Dehydrogenase (U/L)	Serum	8.940± 0.5035	26.50± 1.8708	31.50± 1.8708	33.50± 1.8708
	Gills	2.255± 0.2155	1.890± 0.0626	1.618± 0.0744	1.228± 0.0703
	Muscle	5.130± 0.0660	1.892± 0.0574	1.610± 0.0872	1.240± 0.0687
Total Carbohydrate (mg/g wet weight of tissue)	Liver	63.12± 3.5539	46.31± 1.7532	37.50± 3.3207	23.17± 1.8799
	Heart	52.64± 3.2053	43.93± 2.0671	38.28± 1.8352	29.78± 2.8872
	Kidney	50.04± 1.8102	32.13± 3.1716	23.58± 2.1170	17.91± 0.4851

Average of six values± SD of six observations

Figure 7.1.3 Levels of glucose in the blood of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant increase (P<0.001) in blood glucose level was noted in *O. mossambicus* exposed to the three sub lethal concentrations of ethanol as compared to control group (Figure 7.1.3). Investigations using ANOVA substantiates the above statement and the results are shown below (Table 7.1.3a).

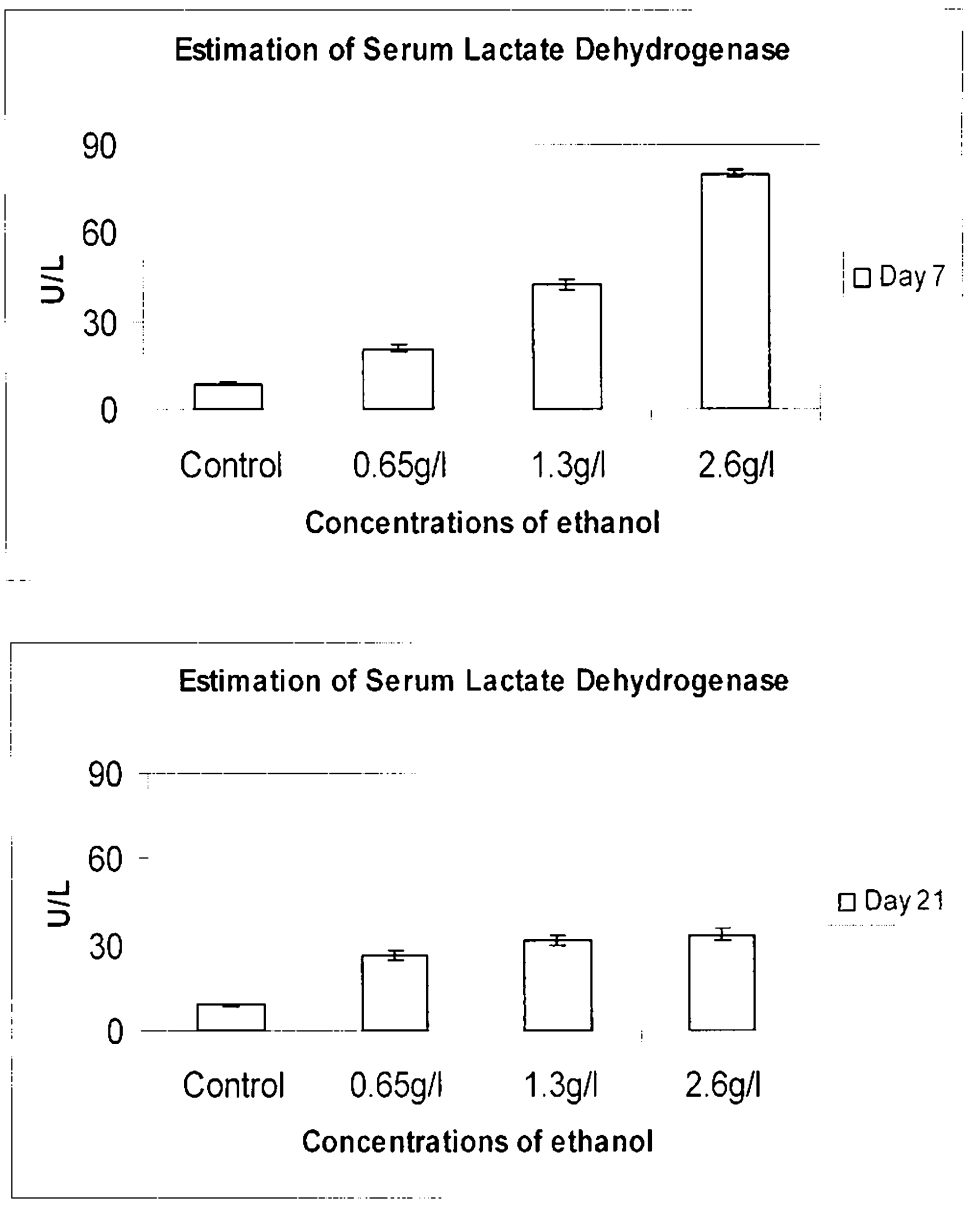
Table 7.1.3a ANOVA table for blood glucose

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	10803.000	1	10803.000	2826.870	0.000
Between Concentrations	32349.009	3	10783.003	2821.637	0.000
Days of exposure × Concentration	8453.684	3	2817.895	737.371	0.000
Error	152.862	40	3.822		
Total	51758.555	47			

df – degrees of freedom

Two Factor ANOVA table revealed that blood glucose levels varied significantly between days (P<0.001). Between concentrations there was a significant difference (P<0.001). Also while taking into consideration the effects of both the days as well as concentrations (Interaction) significant difference (P<0.001) was observed.

Figure 7.1.4 Levels of lactate dehydrogenase activity in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



O. mossambicus exhibited significant ($P<0.001$) increase in the serum lactate dehydrogenase levels when subjected to various sub lethal concentrations of ethanol (Figure 7.1.4). Investigations using ANOVA substantiates the above statement which is shown below (Table 7.1.4a).

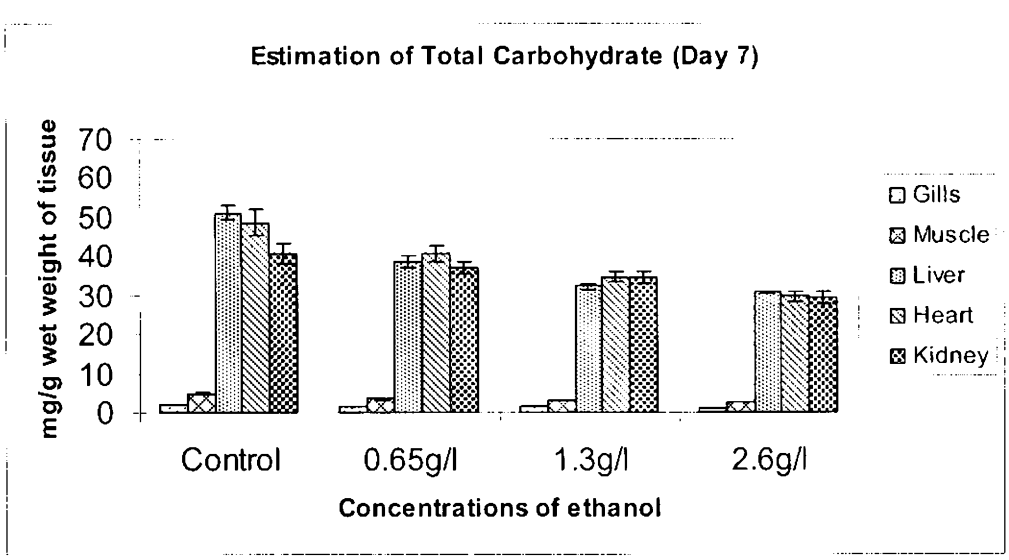
Table 7.1.4a ANOVA table for serum lactate dehydrogenase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	1997.694	1	1997.694	888.960	0.000
Between Concentrations	14909.069	3	4969.690	2211.477	0.000
Days of exposure × Concentration	4950.187	3	1650.062	734.266	0.000
Error	89.889	40	2.247		
Total	21946.839	47			

df – degrees of freedom

Two Factor ANOVA table indicated that serum lactate dehydrogenase levels varied significantly ($P<0.001$) between days. Between concentrations a marked significant difference ($P<0.001$) was noted. In the case of interaction effects of both the days as well as concentrations, also significant difference ($P<0.001$) was seen.

Figure 7.1.5 Levels of total carbohydrate content in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



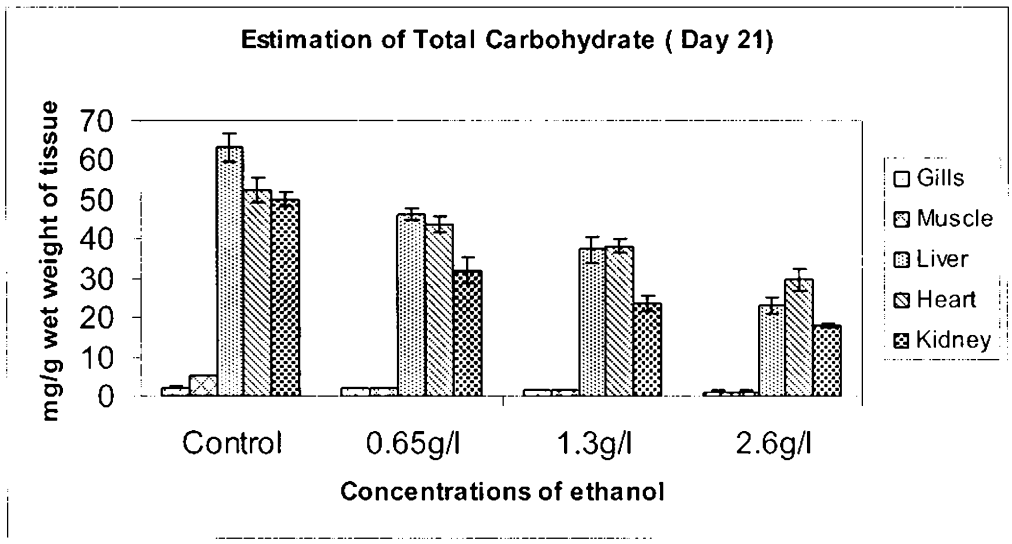


Figure 7.1.5 depicts marked alterations in the total carbohydrate levels in *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure period. Analysis carried out by applying ANOVA supports the above statement (Table 7.1.5a).

Table 7.1.5a ANOVA table for tissue total carbohydrate

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	8.724	1	8.724	0.292	0.589
Between Concentrations	7691.811	3	2563.937	85.880	0.000
Between Tissues	73814.352	4	18453.588	618.108	0.000
Error	6896.490	231	29.855		
Total	88411.378	239			

df – degrees of freedom

Statistical analysis carried out by using Three Factor ANOVA table indicated that, while considering the exposure between days there was no significant difference. But when the comparison was done between concentrations, significant difference ($P<0.001$) was noted. Finally when effect between tissues were compared, then also significant difference ($P<0.001$) was observed.

Table 7.1.6 Multiple Comparison Test (Concentration)

To study further statistical relevance, comparing the significant difference in toxicity induced by different sub lethal concentrations of ethanol with respect to control, multiple comparison test using Dunnett's post hoc test was conducted.

	Groups	Blood Glucose	Serum LDH	Total Carbohydrate
Dunnett's	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001.

Table 7.1.7 Multiple Comparison Test (Tissue)

In order to study all possible pair wise comparisons between tissues multiple comparison tests using Tukey is shown below

	Tissues	Sig.
Tukey	Gills Vs Muscle	0.755[d]
	Gills Vs Liver	0.000[a]
	Gills Vs Heart	0.000[a]
	Gills Vs Kidney	0.000[a]
	Muscle Vs Liver	0.000[a]
	Muscle Vs Heart	0.000[a]
	Muscle Vs Kidney	0.000[a]
	Liver Vs Heart	0.985[d]
	Liver Vs Kidney	0.000[a]
	Heart Vs Kidney	0.000[a]

The values are significant at a = P < 0.001, b = P < 0.01 and not significant at d.

Subsequent comparisons by multiple comparison test using Tukey followed by Dunnett's method is shown above (Table 7.1.6 and 7.1.7)

Subsequently, pair wise comparison between various concentrations with respect to control using Dunnett's method revealed that parameters such as blood glucose, serum lactate dehydrogenase and total carbohydrate content exhibited significant difference (P<0.001).

Subsequent pair wise comparison between various tissues were carried out using Tukey which indicated that muscle tissue when compared with gills exhibited no significant difference (NS), whereas tissues such as liver, heart and kidney when compared with gill tissue exhibited significant difference(P<0.001). Also when liver, heart and kidney tissue were compared with muscle significant difference (P<0.001) was noted. But heart tissue compared with liver exhibited no significant difference (NS). Finally liver and heart tissue when compared with kidney exhibited significant difference (P<0.001).

7.1 D Discussion

Biochemical profiles of blood provide important information about the internal environment of the organism (Masopust, 2000). It can be stated that biochemical parameters that get fluctuated in response to deleterious effects of pollution are ranked as possible biomarkers of pollution. Fish species are widely used to biologically monitor variation in environmental levels of anthropogenic pollutants (Flammarion *et al.*, 2002; Schmitt, 2004; Whyte *et al.*, 2000). Various enzymes can be used as stress indicators or general biomarkers of stress. The role of blood enzymes in monitoring and detecting stress or disease has led to a growing concern in using them as biochemical indicators to trace environmental pollutants (Adham *et al.*, 1997, 1999; William, 1997). The metabolic effects of ethanol are mainly brought about either by the direct action of ethanol or by its metabolites. Ethanol causes hyperglycemia or hypoglycemia depending on whether the glycogen stores are adequate or not.

Different blood parameters are often subjected to change depending upon the stress condition and various other environmental factors (Goel *et al.*, 1981). In the present study a significant increase (P<0.001) (Fig 7.1.3) in blood glucose was cited when *O. mossambicus* was exposed for 21 days with a periodical sampling at 7 days

under varying concentrations of ethanol. The increase in blood glucose level was mainly due to the response of exposed fishes to metabolic stress. The findings of Simon *et al.* (1983) support the present observation. The changes observed in blood glucose content were found to be concentration dependent. Fernandes *et al.* (2008) also observed an increase in blood glucose level in mullets in a highly polluted water body. Another possible reason for the increase in blood glucose level may be due to severe nephritis and hepatic disorder which in turn is caused due to the enhanced breakdown of liver glycogen leading to disruptions in carbohydrate metabolism. The findings of Wedemeyer and McLeay (1981) also supported the present finding as high levels of blood glucose are caused by disorders in carbohydrate metabolism mainly arising due to physical and chemical stress. Similar trends were also found in teleost fishes when exposed to distillery and industrial effluents (Shaffi, 1980, 1981). Increase in blood glucose level thereby indicates increased breakdown of glycogen to glucose and its mobilization to other tissues to meet the energy crisis. Another possible reason for the increase in blood glucose level could be the decrease in glycogen synthesizing potential of the tissues which mainly arises as a consequence of cellular damage. Findings of Jehosheeba and Babu Philip (2006), who also observed increase in blood glucose levels in *O. mossambicus* when exposed to water accommodated fractions (WAF) of Bombay high crude oil for 21 days support the present result. The observations of Maria *et al.* (2004) where similar increase in blood glucose level was noticed in largemouth bass sampled from effluent contaminated streams, is also in agreement with the present finding.

Carbohydrate metabolism is a major source of energy production in many fish species and the activity of LDH has been considered as an easy target for the action of various xenobiotics. Lactate dehydrogenase (LDH) is referred to as an enzyme usually present in all tissues. It has been described as a reliable early-warning indicator of cellular stress. It also forms the center of a delicately balanced equilibrium between catabolism and anabolism of carbohydrates (Everse and Kaplan, 1973). A significant increase ($P<0.001$) (Fig 7.1.4a) in serum LDH was observed when *O. mossambicus* was exposed to different sub lethal concentrations of ethanol for 21 days with an intermediate sampling at 7 days. An increase in serum LDH activity observed when *O. mossambicus* was exposed to different concentrations

of ethanol for 7 days points out to the high oxygen demand which can be a causative factor to induce the anaerobic oxidation to release energy by enhancing serum LDH activity. This observation was in turn supported by Saha *et al.* (1999) who too observed similar trend in *O. mossambicus* when exposed to phenol. Similar findings were cited by Khadiga *et al.* (2002) who too have reported an increase in serum LDH in *O. niloticus* (L.) in response to ambient water pollution. Sadakat Ozdil *et al.* (2004) also observed an increase in serum LDH when rats were treated with ethanol for 3 days. Therefore it can be inferred that the increase in LDH activity in serum could be an indicator of liver destruction brought about by ethanol resulting in the release of the enzyme into the blood. It can also be correlated with the elevated rates of lipid peroxidation. Mitchell *et al.* (1980) have observed an increased release of LDH into the serum which has been referred to as an indication of damage to the tissues which points out to the integrity of cell membranes. The increase in the activity of the above mentioned enzyme in serum was directly proportional to the degree of cellular damage. Increased activity of LDH observed upon exposure to 7 and 21 days to ethanol indicates that the fish, *O. mossambicus* switched on to anaerobic respiration mainly to meet the energy demands when aerobic oxidation was lowered. This also suggested that the forward reaction of LDH, namely pyruvate to lactate, may be more operative during exposure to ethanol. The findings of Jayantha Rao *et al.* (1987) supported this view. They have observed similar increase in serum LDH activity in *Tilapia mossambica* upon chronic exposure to heptachlor. Dezwaan and Zandee (1972) have also reported that anoxic or hypoxic conditions were known to elevate carbohydrate consumption in *Mytilus edulis*. Dampure (1984) also stated that injury to heart, liver, kidney and lungs in contaminated *O. niloticus* could be forecasted by monitoring the LDH levels in various body fluids. Besides alterations that are genetically based, diseases may affect enzymes by damaging the tissues and result in the leakage of the enzymes into the surrounding body fluids as mentioned by William (1997).

Carbohydrates represent the principal and immediate energy precursors for organisms exposed to stress (Umminger, 1970). It has been shown that pollutants induce hypoxic conditions, which result in the extra expenditure of carbohydrate constituents. This statement was put forth by Dhavale and Masurekar (1986). Total

carbohydrate content was decreased in tissues such as gills, muscle, liver, heart and kidney of *O. mossambicus* upon exposure to sub lethal concentrations of ethanol for 21 days with periodical sampling at 7 days (Table 7.1.1, Table 7.1.2 and Figure 7.1.5). Total carbohydrate content in the tissues was found to decrease with increase in both the concentration of ethanol and duration of exposure. This explains the utilization of excess energy needed to cope with stress induced by ethanol. Anoxia or hypoxia increases carbohydrate consumption (Dezwaan and Zandee, 1972) and thereby induces a sort of respiratory stress on organisms even at sub lethal levels resulting in additional expenditure of energy. The increased glycogenolysis indicated a general disturbance in carbohydrate metabolism, which might have an adverse effect on the life of exposed animals (Dhavale *et al.*, 1988). From the above trend, it can be easily visualized that there is a rapid utilization of total carbohydrates by all tissues to meet higher energy demands to mitigate the stress caused by ethanol. Jehosheeba and Babu philip (2006) support the present finding by way of their results in which similar decrease in the carbohydrate content was noticed in the tissues of *O. mossambicus* exposed to water accommodated fractions (WAF) of Bombay high crude oil for 21 days.

Another possible reason for the decrease in carbohydrate content in the tissues of *O. mossambicus* may be due to the rapid utilization of carbohydrates by the tissue, possibly to overcome the stress. Findings of Amudha *et al.* (2002) showed a decrease in carbohydrate content in the tissues of *O. mossambicus* exposed to sub lethal concentrations of dairy effluent. In control fish tissues, liver showed the highest total carbohydrate content followed by heart and kidney tissues. The total carbohydrate content in gills and muscle tissues was very low. This shows that liver is the major site for synthesis and storage of carbohydrates in fish. The observed low levels of total carbohydrates in the gill tissues depict low glycogen synthetic potentials. It may be suggested that stress conditions created by effluent stimulates the adrenal glands to liberate epinephrine which in turn would elevate the level of glucose. This hypothesis has been proved to be right in the case of some fishes subjected to environmental stress (Silbergeld, 1974). It is well established that hyperglycemia induced in fish is due to hypoxic condition (Bansal *et al.*, 1979; Sastry and Siddique, 1982). Gopal *et al.* (1980) suggested that the disrupted

carbohydrate metabolism may be due to the enhanced breakdown of liver and muscle glycogen (glycogenolysis). Similar trend was also cited by David and Ray (1966) in fresh water fish *Cyprinus carpio* exposed to different sub lethal distillery effluent concentrations for 7 and 21 days. Thus the toxicological impact on different tissues of *O. mossambicus* suggests the tendency of the metabolism of carbohydrates to shift more towards anaerobic dependence than aerobic oxidation through Kreb's cycle.

7.2 EFFECT OF ETHANOL ON MITOCHONDRIAL ELECTRON TRANSPORT CHAIN

Contents

7.2A Introduction
7.2B Materials and Methods
7.2B.1 Preparation of tissue samples for experiments
7.2B.2 Method used for biochemical analysis
7.2C Results
7.2D Discussion

7. 2A Introduction

Cell representing the smallest unit of an organism integrates all functions of life such as metabolism, growth, movement, sensitivity and reproduction. Therefore any scientific study dealing with the biological effects of xenobiotics should focus on the cell. Cytochromes are most frequently studied in relation to their role in the metabolism of xenobiotics (Stoilov *et al.*, 2001). Entering the body of an organism, xenobiotics bind to specific cellular structures called receptors that are localised on the cell surface or inside the cell either in its cytoplasm or on cell organelles. The binding of a xenobiotic with its receptor may induce biochemical processes that have toxic or other adverse effects on the cell. In macro organisms, these processes subsequently affect organs, the organism itself and eventually the whole population.

Mitochondrial cytochrome c oxidase (E.C 1.9.3.1) is a key enzyme of the respiratory chain. Cytochrome oxidase or Cytochrome c oxidase (COX) or complex IV is the last enzyme of the electron transport chain and like complex I and II, is located in the inner mitochondrial membrane. Mitochondria are the intracellular organelles responsible for ATP synthesis through the coupling of oxidative phosphorylation to respiration in human and animal cells. Sohal and Sohal (1991) have cited mitochondria as the major intracellular source during oxidative

phosphorylation. Cytochrome c oxidase is partly encoded by the nuclear genome (10 sub units) and partly by mitochondrial DNA (3 sub unit). In *in vivo* conditions it transfers electrons from Cyt.c to oxygen so that together with protons water is formed. Cyt.c must continuously be reoxidized to have a continuous transport of electrons (Jan *et al.*, 2004). In aerobic organisms, mitochondrial respiration (oxidative phosphorylation) is the dominant means of ATP production. Aerobic cells contain two kinds of DNA, nuclear and mitochondrial, and both are required for the construction of the enzymes involved in oxidative phosphorylation. Mitochondria synthesize most of the adenosine triphosphate (ATP) needed by mammalian cells. Oxidation of various substrates results in the formation of reduced nicotinamide adenine dinucleotide (NADH) or reduced ubiquinone, which are oxidized through the electron transport chain in the inner mitochondrial membrane. The associated extrusion of protons from the mitochondrial matrix generates a pH gradient and an electrical potential difference across the inner mitochondrial membrane, which together constitute the proton motive force which is said to be the driving force for ATP synthesis through the proton pumping F_1F_0 adenosine triphosphatase (ATP synthase).

The fish, as a bioindicator species, plays an increasingly important role in the monitoring of water pollution because it responds with great sensitivity to changes in the aquatic environment. The effects of exposure to sub lethal levels of pollutants can be measured in terms of biochemical, physiological or histological responses of the fish organism (Mondon *et al.*, 2001). The present investigation was focused to determine the levels of cytochrome c oxidase in gills, muscle, liver, heart and kidney tissues of *O. mossambicus* when subjected to different sub lethal concentrations of ethanol for suitable exposure periods.

7. 2B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design remains the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

7. 2B.1 Preparation of tissue samples for experiments

Electron transport chain studies were carried out in *O. mossambicus* weighing 10 ± 2 g. After exposing the fishes for 7 and 21 days to different sub lethal concentrations of ethanol, the fishes were killed by ordinary pithing (by damaging the brain and severing the spinal cord between the head and the trunk region using a sharp needle) and the tissues such as gills, heart, kidney, liver and muscle were removed from its body. They were then washed in ice cold 0.33 M sucrose and dried using blotting paper in order to remove blood and other body fluids. 10% homogenate of gill and muscle followed by 2% homogenate of liver and 0.5% homogenate of heart and 1% homogenate of kidney tissues were prepared in 0.1M cold phosphate buffer of pH 7.6. The homogenates were centrifuged at 1000g for 15 minutes. The supernatant was taken. They were stored at -20^{0}C and thawed prior to analysis.

7.2B.2 Method used for biochemical analysis

Estimation of Cytochrome c oxidase (Ferrocytochrome c: Oxygen oxidoreductase; E.C 1.9.3.1)

Principle

Cytochrome-c-oxidase activity was estimated by determining the rate of oxidation of reduced ferricytochrome c using method described by Oda *et al.* (1958).

Reagents

1. 0.1M Phosphate buffer (pH 7.6)
2. 0.2 M ρ-phenylenediamine
3. 0.2% Neotetrazolium chloride
4. 10^{-4} M Cytochrome c
5. 1:1 Ether: acetone mixture

Procedure

Into a test tube 0.2 ml of p-phenylenediamine, Neotetrazolium chloride, cytochrome-c and 0.2 ml of the enzyme were added. This constituted to a total volume of 0.8 ml of reaction mixture. The tubes were then mixed well and were incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 0.4 ml of 1 N H_2SO_4. The tubes were then kept overnight. The yellowish brown precipitate formed was the oxidized product of p-phenylenediamine, which was extracted in 5.0 ml of 1:1 ether: acetone mixture. The absorbance obtained was measured in a spectrophotometer at 520 nm against ether: acetone (1:1) mixture served as blank. The enzyme activity was expressed in nanomoles of formazan formed/mg protein/h.

7. 2C Results

Effect of different sub lethal concentrations of ethanol exposed for 21 days with a periodical sampling at 7 days on cytochrome –c-oxidase activity in the gills, liver, kidney, heart and muscle tissues of *O. mossambicus* are given in Tables 7.2.1 to 7.2.5 and in figure 7.2.3. The statistical analysis of the results were carried out by Three factor ANOVA (Analysis of Variance) followed by Tukey's test and Dunnett's method.

Table 7.2.1 Effect of exposure to different concentrations of ethanol for 7 days on the levels of cytochrome-c-oxidase activity in different tissues of *O. mossambicus*.

Parameter Investigated	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Cytochrome c oxidase	Gills	2.377± 0.2148	1.575± 0.0394	1.018± 0.0492	0.905± 0.0730
	Muscle	1.572± 0.0232	1.240± 0.0566	0.835± 0.0243	0.649± 0.0319
	Liver	0.388± 0.0618	0.289± 0.0037	0.167± 0.0137	0.120± 0.0078
	Heart	0.502± 0.0147	0.416± 0.0052	0.236± 0.0395	0.083± 0.0277
	Kidney	0.574± 0.0168	0.468± 0.0264	0.349± 0.0124	0.196± 0.0206

Values are expressed as nanomoles of formazan formed/mg protein/hr
Average of six values± SD of six observations

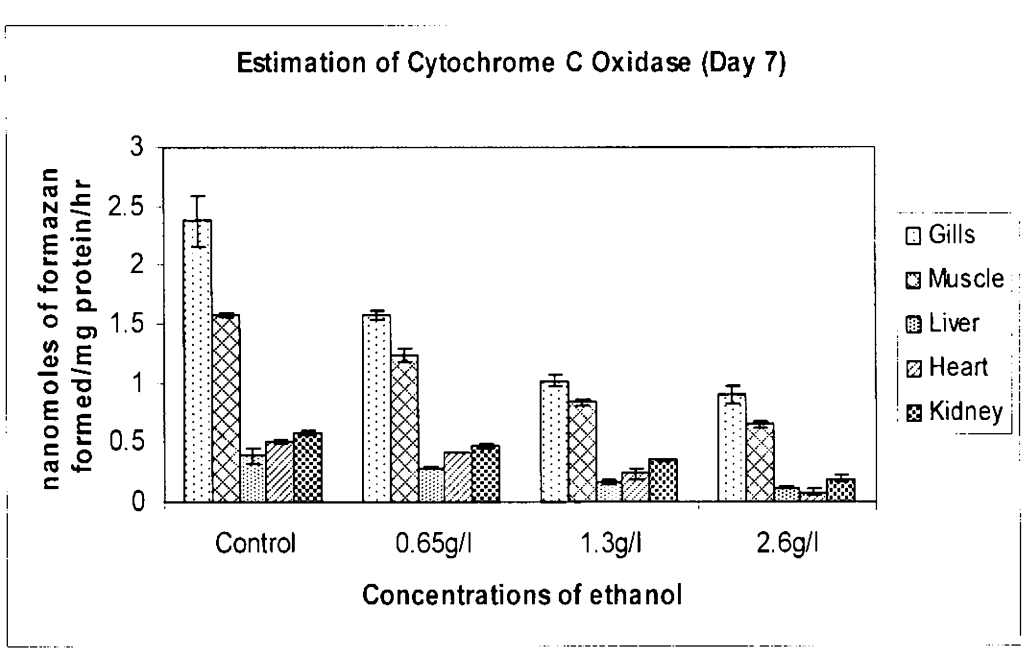
Table 7.2.2. Effect of exposure to different concentrations of ethanol for 21 days on the levels of cytochrome-c-oxidase activity in different tissues of *O. mossambicus*.

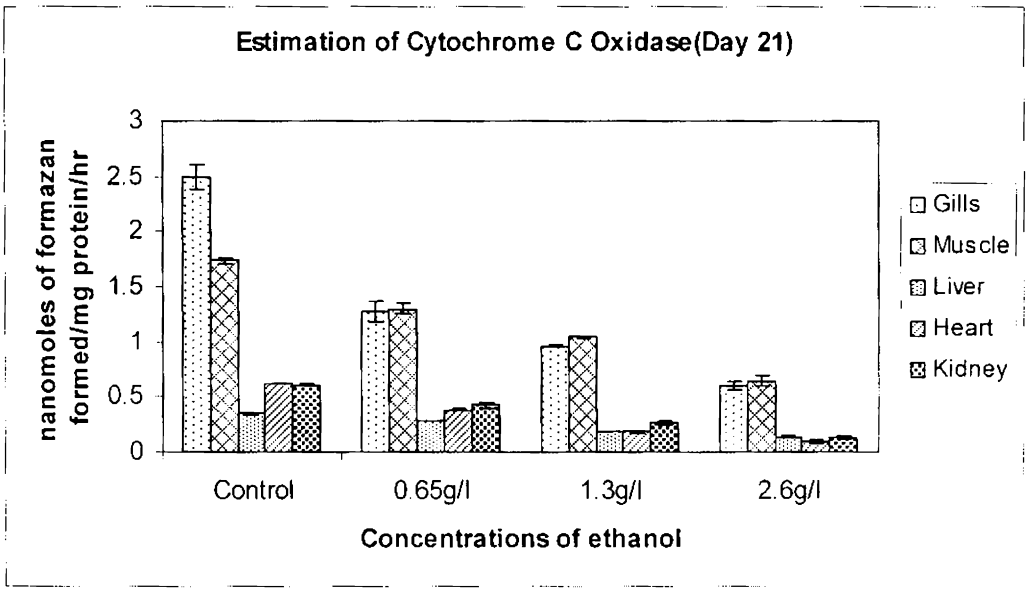
Parameter Investigated	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Cytochrome c oxidase	Gills	2.492± 0.1127	1.270± 0.0894	0.957± 0.0100	0.605± 0.0338
	Muscle	1.738± 0.0264	1.302± 0.0542	1.045± 0.0105	0.641± 0.0498
	Liver	0.351± 0.0055	0.284± 0.0044	0.192± 0.0052	0.141± 0.0129
	Heart	0.618± 0.0064	0.382± 0.0056	0.181± 0.0056	0.094± 0.0111
	Kidney	0. 607± 0.0113	0.424± 0.0302	0.264± 0.0171	0.129± 0.0157

Values are expressed as nanomoles of formazan formed/mg protein/hr

Average of six values± SD of six observations

Figure 7.2.3 Variations in cytochrome-c-oxidase activity in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Cytochrome c oxidase activity exhibited marked changes in the tissues such as gills, muscle, liver, heart and kidney of *O. mossambicus* when exposed for 7 and 21 days to various sub lethal ethanol concentrations with respect to control (Figure 7.2.3). Statistical analysis using ANOVA corroborated the above statement (Table 7.2.3a).

Table 7.2.3a Three Factor ANOVA table for tissue cytochrome-c-oxidase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.009	1	0.009	0.179	0.672
Between Concentrations	19.852	3	6.617	133.690	0.000
Between Tissues	54.548	4	13.637	275.516	0.000
Error	11.434	231	0.049		
Total	85.843	239			

df – degrees of freedom

Three Factor ANOVA table revealed that between days of exposure no significant difference was obtained. Between concentrations significant difference ($P<0.001$) was obtained. While considering the effect between tissues significant difference ($P<0.001$) was noted.

Table 7.2.4 Multiple Comparison Test (Concentration)

Subsequent comparisons by multiple comparison tests between concentrations using Dunnett's method is shown below.

	Groups	Sig.
Dunnett	Control Vs 0.65g/l	0.000[a]
	Control Vs 1.3g/l	0.000[a]
	Control Vs 2.6g/l	0.000[a]

The values are significant at a = P < 0.001.

Table 7.2.5 Multiple Comparison Test (Tissue)

Subsequent comparisons by multiple comparison tests between tissues using Tukey is shown below

	Tissues	Sig.
Tukey	Gills Vs Muscle	0.000[a]
	Gills Vs Liver	0.000[a]
	Gills Vs Heart	0.000[a]
	Gills Vs Kidney	0.000[a]
	Muscle Vs Liver	0.000[a]
	Muscle Vs Heart	0.000[a]
	Muscle Vs Kidney	0.000[a]
	Liver Vs Heart	0.501[d]
	Liver Vs Kidney	0.027[c]
	Heart Vs Kidney	0.643[d]

The values are significant at a = P < 0.001, c = P < 0.05 and not significant at d.

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. Taken into consideration the cytochrome-c-oxidase activity significant decrease (P<0.001) was observed in all the three sub lethal concentrations of ethanol such as 0.65 g/l, 1.3 g/l and 2.6 g/l with respect to control. For considering the pair wise comparison between tissues, Tukey

was taken into account. This revealed that when tissues such as gills and muscle were compared with liver, kidney and heart significant difference ($P<0.001$) was obtained. Similarly when liver was compared with heart no significant difference (NS) was got. Where as liver when been compared with kidney significant difference ($P< 0.05$) was observed. Heart when being compared with kidney also exhibited no significant difference (NS).

7.2 D Discussion

Mitochondria carry out a variety of functions, of which oxidative phosphorylation is the most important. Detoxification of oxygen via its reduction into H_2O by the cytochrome oxidase system takes place in the mitochondria. Cytochrome c oxidase and NADH dehydrogenase are the enzymes involved in the electron transport chain and are located in the inner mitochondrial membrane. Their role is ultimately linked to the production of useful energy rich compounds such as ATP. The respiratory process involves the transport of electrons via cytochromes to molecular oxygen. Under normal conditions, the rate of oxidation of NADH through the electron transport chain is often constrained by the cellular demand for ATP. One consequence of cytochrome oxidase inhibition is the reduced yield of ATP (Wong-Riley, 1989). The lower capacity for oxidative phosphorylation further stresses the balance between the reducing pressure imposed by ethanol and the capacity for oxidative phosphorylation (Jan *et al.*, (2002). Sohal and Sohal (1991) has cited mitochondria as the major intracellular source for electrons during oxidative phosphorylation. Mitochondria have been referred to as the primary target of reactive oxygen species (ROS), which are generated under normal conditions as by-products of aerobic metabolism in animal and human cells. It has been established by stating that defects in the respiratory chain lead to enhanced production of ROS and free radicals in mitochondria, resulting in mitochondrial DNA mutations which indirectly impair glucose sensing by reducing intracellular concentrations of ATP (Shoffner and Wallace, 1994) which serves as an important metabolic fuel. Inhibition of electron transport in mitochondria can also lead to production of ROS.

Ethanol brings about an increase in reactive oxygen species in which mitochondria plays a prominent role. The ability of ethanol to increase mitochondrial ROS production is linked to its metabolism via oxidative processes and/or ethanol related alterations to the mitochondrial electron transport chain. Variations in cytochrome concentrations may affect the transport of electrons via the transport chain and thereby alter the energy production of mitochondria. It has been reported that reduction in functioning of mitochondrial enzymes may be related to a defect in the mitochondrial energy production that would impair protein synthesis and energy production. A decrease in mitochondrial cytochrome content could result in the concomitant loss of oxidative phosphorylation capacity (Shultz and Chan, 2001). The findings of Lemasters *et al.* (1999) and Volbracht *et al.* (1999) also indicated a tissue-specific decrease of ATP which contributes to the preferential destruction of nerve and skeletal muscle cells via necrosis and/or apoptosis.

Mitochondrial dysfunction is an important mechanism of ethanol induced toxicity. Results indicated that exposure of *O. mossambicus* to varying sub lethal concentrations of ethanol for 7 and 21 days, brought about marked inhibition in the Cyt.c oxidase activity in the different tissues of *O. mossambicus* (Table 7.2.1 to 7.2.3 and Figure 7.2.3a). Inhibition in cytochrome c oxidase has been reported in the myocardium of young rats when being treated with Aminoacetonitrile for 5 days (Clemmons and Jackson, 1962). Ethanol promotes oxidative stress, both by increasing ROS formation and by decreasing cellular defense mechanism.

Ethanol damages the permeability of inner mitochondrial membrane which in turn inhibits the expression of some components of the Mitochondrial electron transport chain (METC) such as NADH dehydrogenase and Cytochrome c oxidase, promoting ROS formation and resulting in decreased ATP synthesis (Frank, 2003). Cyt. c oxidase is a haemoprotein, and acts as a terminal component of the respiratory chain in mitochondria. It transfers electrons to the final acceptor, oxygen. Thus being a terminal link in electron transfer system (ETS) it produces ATP molecules thereby influencing other cellular metabolic process. Decrease in Cyt. c oxidase activity might be either due to the result of reduced availability of oxygen, which in turn has reduced the capacity of the electron transport system to produce ATP

molecules or it can be due to the direct impact of the toxicants. A decrease in cytochrome oxidase activity in muscle and heart tissues of rats were observed when treated with sodium azide for 28 days (Jason *et al.*, 2001). Furthermore, chronic ethanol treatment affects mitochondrial oxidative phosphorylation in the tissues by suppressing the synthesis of protein subunits that are encoded on mitochondrial DNA (mt DNA) as stated by Cunningham *et al.* (1990) and Cahill and Cunningham (2000). These include subunits of the main respiratory complexes, NADH dehydrogenase (Complex I), cytochrome b-c (Complex III), and cytochrome oxidase (Complex IV), as well as the ATP synthase complex (Complex V). Furthermore, cytochrome oxidase inhibition increases reactive oxygen species (Partridge *et al.*, 1994). Agents such as nitric oxide, hydrogen sulfide, and cyanide, present in certain environments, produce effects similar to that of ethanol in rats (Heales *et al.*, 1999), which seems to be relevant to environmental health. Some toxic agents may also cause a decrease in an enzyme activity by directly inhibiting its catalytic activity (Jason *et al.*, 2001). The decrease in cytochrome c oxidase activity as indicated by Sudhanshu and Ajay (2004) in liver and muscle tissues of *Channa punctatus* after exposing them to sub lethal doses of alcoholic extract of N. indicum leaf for 96 hours also supports the present study.

Ethanol induced damage to mitochondrial DNA, if not adequately repaired, impairs mitochondrial function which further increases oxidative stress in the cell, leading to a vicious cycle of cell damage. Uncontrolled mitochondrial formation of ROS promotes the inappropriate activation of the mitochondrial permeability transition, increasing the sensitivity of cells which ultimately leads to proapoptotic or damage cells. In combination with ethanol induced defects in mitochondrial function, these alterations may promote both apoptotic and necrotic cell death. The present study was carried out to investigate the effect of ethanol on cytochrome c oxidase activity in the tissues of *O. mossambicus*.

7.3 EFFECT OF ETHANOL ON PROTEIN METABOLISM

Contents

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h. Estimation of acid phosphatase
7.3C Results
7.3D Discussion

7.3A Introduction

It is a well known fact that water quality conditions are constantly being threatened by pollution. Most of the industrial effluents often contain pollutants toxic to the biota of the receiving stream. Numerous works have been carried out on the assessment of quality of fresh water pollution by the discharge of effluents from the industries. Most of the effluents are strongly biotoxic in nature due to its high alkalinity, BOD, COD and alarmingly low DO content (Baruah *et al.*, 1996 b). Environmental stressors both natural and anthropogenic cause changes in cellular function which alter the physiology of organ systems in the fish (Rajeev *et al.*, 2007).

Fishes being sensitive to contaminants, suffer from physiological and biochemical damages when these contaminants enter the organs of these animals (Nemcsok *et al.*, 1987). The harmful effects especially sub-lethal of the pollutants retard growth and adversely affect the metabolic activities of the surviving individuals (Nemcsok and Boross, 1982; Weis and Weis, 1977). According to Musa and Omoregie (1999), fishes are intimately associated with the aqueous

environment, and physical and chemical changes in the environment are rapidly reflected as measurable physiological changes in fish. As fish fauna serves as a food source for man it is essential to know the impact of water pollution on these organisms. The physiological and biochemical changes in fish were recorded by Gill and Pant (1981) and by Amudha and Mahalingam (1999).

The polluted aquatic environment induces severe physiological and biochemical stress leading to impairment of major metabolic pathways including nitrogen metabolism in fishes. Nitrogen metabolism is not lower in importance than carbohydrate and lipid metabolism. Proteins make up the structural tissue for muscles and tendons, transport oxygen (e.g. haemoglobin), catalyze all biochemical reactions as enzymes, and regulate reactions as hormones. Proteins in excess are used to supply energy. They also build reserves of glucose, glycogen, or lipids. Changes occurring in the biochemical characteristics of fishes provide a sensitive measure to know the health of fish fauna (Nair *et al.*, 1984). Biochemical constituents and enzymes have been explored, as potential biomarkers for a variety of organisms. They were the first detectable/quantifiable responses to environmental changes, serving as markers for both exposure and effect in organisms (Vijayavel *et al.*, 2006). Several specific enzymes have been proposed for monitoring water pollution (Agradi *et al.*, 2000). Fish blood is an important tissue of the body, which performs most of the vital activities of the life (Saxena and Sharma, 1979). Although teleost fishes predominantly excrete ammonia as a nitrogenous waste, a small amount of urea, usually around 10 - 15% of the total nitrogenous wastes, is also excreted by most teleosts (Saha and Ratha, 1998; Wood, 1993). The formation of urea in most teleosts is thought to result from the breakdown of dietary arginine and/or uric acid (Mommsen and Walsh, 1991; Wright *et al.*, 1995; Walsh, 1998). In ammoniotelic teleosts, urea is produced mainly by uricolysis (Goldstein and Forster, 1965; Wood, 1993). Acid phosphatase is referred to as the hydrolytic enzyme which plays an active part in the dissolution of dead cells in the body. Stimulation or inhibition of these enzymes usually results in the disturbance of metabolism (Saxena *et al.*, 1982). In the present study, assays were carried out to measure the levels of albumin, urea, uric acid, creatinine, ammonia, free amino acids, protein and the enzyme acid phosphatase in the serum and also in gills, muscle, liver, heart and

kidney tissues of *O. mossambicus, on* exposure to different sub lethal concentrations of ethanol.

7.3B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

7.3B.1 Preparation of serum samples for experimental studies

The method remains the same as described in chapter 5, section 5.1B.1.

7.3B.2 Experimental procedures

The following were the parameters studied under protein metabolism.

a. Estimation of albumin

Principle

Albumin in serum was estimated by the method of Bartholomew *et al.* (1966). Albumin present in serum binds specifically with the dye, bromocresol green at pH 3.8 to form green coloured complex, which brings about a change in the wave length with maximium absorption. This in turn brought about a change in the spectral profile of the dye at 637 nm. The intensity of the colour was directly proportional to the albumin concentration.

Reagents

1. 1M sodium citrate: 29.4 g of sodium citrate was dissolved in 100 ml water.
2. 1M Citric acid: 21 g citric acid was dissolved in 100 ml water.
3. 0.01 M Bromocresol green (BCG): 0.0698 g of BCG was dissolved in 0.98 ml of 0.1 M sodium hydroxide. It was then made up to 10 ml with water.

4. Buffering agent: To about 800 ml water, 17.3 ml of 1M sodium citrate, 32.7 ml of 1 M citric acid and 6 ml 0.01M BCG were added. The contents were then diluted to 1L with dist. H_2O. The pH was adjusted to 3.8.
5. Standard bovine serum albumin solution: 250 mg of bovine serum albumin was made up to 25 ml with 0.9% saline. 20 ml of the stock standard was made up to 100 ml with water.

Procedure

To 0.02 ml of serum sample, 0.05 ml of distilled water was added. To this 4 ml of buffering reagent was added. The contents were mixed well. Similarly standards of volume ranging from 0.1 to 0.5 ml were taken. It was then made up to 0.5 ml with water. 0.5 ml of water was taken as blank. 4 ml of buffering reagent was added to all the tubes and they were kept for 10 minutes. The colour obtained was then read at 637 nm. The values obtained were expressed as g/dl of serum.

b. Estimation of urea

Principle

The method employed was that of Varley (1976) in which, diacetyl monoxime in the presence of acid, hydrolyses to produce the unstable compound diacetyl. This diacetyl reacts with urea to produce a yellow diazine derivative. The colour of this product becomes pink by the addition of thiosemicarbazide which was measured colorimetrically at 520 nm.

Reagents

1. TCA, 10%
2. Stock Diacetylmonoxime, 25 g/l
3. Stock Thiosemicarbazide, 2.5 g/l
4. Acid ferric chloride solution: Added 1.0 ml sulphuric acid to 100 ml of ferric chloride solution.

5. Acid reagent: Added 10 ml of Ortho phosphoric acid, 80 ml sulphuric acid and 10 ml acid ferric chloride solution to 1 litre of water. The contents were then mixed well.
6. Colour reagent: To 300 ml of acid reagent, 200 ml of water, 10 ml of stock diacetylmonoxime and 2.5 ml of thiosemicarbazide solution was added.
7. Stock urea standard: 5 to 50 mmol/L of pure urea was taken which contained concentration ranging from 30 to 300 mg/L.

Procedure

Into a test tube 0.2 ml of serum, 1.0 ml of water and 1.0 ml of 10% TCA were added. The contents were mixed well and centrifuged. 0.2 ml of the supernatant was taken. To this added 3.0 ml of colour reagent. At the same time 0.2 ml of water as blank and 0.2 ml of standard urea solution were taken. All the tubes were heated in a boiling water bath for 20 minutes. The tubes were cooled to room temperature and the colour developed was then read at 520 nm within 15 minutes. The result was expressed as mg/dl of serum.

c. Estimation of uric acid

Principle

Uric acid present in the serum was estimated by the method of Caraway (1955, 1963). Uric acid was oxidised to allantoin and carbondioxide by phosphotungstic acid reagent in alkaline solution. Phosphotungstic acid thus obtained was reduced in this reaction to tungsten blue which was measured at 660 nm.

Reagents

1. Phosphotungstic acid reagent
2. 10% Sodium carbonate
3. Standard uric acid: 100 mg of uric acid and 60 mg of lithium carbonate were taken. It was then dissolved in about 50 ml of distilled water. This was

heated to about 60°C to dissolve the uric acid completely. After cooling the solution was finally made up to 100 ml with water.

4. Working standard: Diluted 1.0 ml of stock to 10 ml with water. 1.0 ml of this solution contains 100 µg of uric acid.

Procedure

To 0.1 ml of the serum sample, 2.9 ml of water was added followed by 0.6 ml each of phosphotungstic acid and sodium carbonate. A blank was set up with 3.0 ml of dist. H_2O. A set of standards were also treated in the same manner. The color obtained was then read at 640 nm after 10 min. The values were expressed as mg/100 ml of serum.

d. Estimation of creatinine

Principle

Creatinine content was estimated by modified Jaffe's method without deproteinization (Owen *et al.*, 1954). Creatinine forms a coloured complex with picrate in alkaline medium. The rate of formation of the complex formed was measured at 540 nm.

Reagents

1. Picric acid: 8.02 g/litre
2. NaOH: 12.8 g/litre
3. Reagent mixture: Mixed one part by volume of dilute NaOH with one part by volume of picric acid at least 30 minutes before the assay.
4. Standard creatinine: Dissolved 100 mg of creatinine in 100 ml of distilled water.
5. Working standard: 2.0 ml of the stock was diluted to 100 ml. This contained 20 µg of creatinine/ml.

Procedure

Pipetted out 0.2 ml of serum and 2.0 ml of the reagent mixture into a cuvette. Simultaneously, a blank was set up with the reagent mixture and distilled water. The contents were mixed well and the change in absorbance was measured after 30 sec, which was taken as A1 and exactly after 2 min, the absorbance was read as A2 at 490 nm. A set of standards were also treated in the same manner. A2-A1 gave the change in absorbance which was mainly used to measure the creatinine content present in the sample. The result obtained was expressed as mg/dl of serum.

e. Estimation of ammonia

Principle

Ammonia in serum sample was estimated using the method of Boltz and Howel (1978). Sodium hypochlorite (NaOCl) combines with ammonia present in the sample to produce NH_2Cl and OH^- ions. Sodium nitroprusside catalyses the reaction. NH_2Cl in the presence of $3OH^-$ combines with phenol and forms a quinonoid complex. This quinonoid complex combines with another phenol molecule to form indophenol, a coloured compound. The colour thus formed is directly proportional to the ammonia present in the sample.

Reagents

1. Reagent A: Dissolved 10 g of phenol with 50 mg of sodium nitroprusside in 500 ml of Dist. H_2O. (This solution is stable for 1 month if kept in stoppered amber coloured bottle in a refrigerator).
2. Reagent B: Dissolved 5 g of NaOH in 10 ml of sodium hypochlorite and diluted to 500 ml with Dist H_2O.
3. Standard ammonia solution: Dissolved 0.3819 g of anhydrous ammonium chloride in 1 litre of H_2O
4. Working ammonia solution: Diluted 1 ml of stock solution to 1000 ml with water
5. De proteinizing agent: 80% ethanol

Procedure

To 0.2 ml of serum, 2 ml of deproteinizing agent was added. It was then centrifuged at 5000 rpm for 5 minutes. Supernatant was taken. To 1 ml of the supernatant, 2.5 ml of reagent A was added. Similarly in the case of blank, 1 ml of 80% ethanol was added followed by the addition of 2.5 ml of reagent A. A set of standard ammonia solutions were also treated similarly. All the tubes were kept for 5 minutes at room temperature. To each tube 2.5 ml of reagent B was added. The tubes were again kept for 5 minutes at room temperature. After five minutes all the tubes were incubated at 37^0C for 20 minutes. The optical density was read after 30 minutes at 625 nm. The values were expressed as mg%.

f. Estimation of total protein

Total protein content in tissues was estimated by the method of Lowry *et al.* (1951) as described in chapter 5,Section 5.1B.2h.

Procedure

10% homogenates of gill and muscle, 5% homogenate of liver, and 1% homogenates of heart and kidney tissues were prepared in 0.33M cold sucrose solution. The homogenates were centrifuged. Protein assay was conducted using these homogenates as described in chapter 5, Section 5.1B.2h and the results obtained were expressed as mg/g wet weight of tissue.

g. Estimation of free amino acids (Ninhydrin Positive Substances)

Principle

Total free amino acids also known as Ninhydrin positive substances were estimated by the method of Moore and Stein (1954). Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha-amino acids and yields an intensely coloured bluish purple product which is colorimetrically measured at 570 nm.

Ninhydrin + alpha-amino acid ⟶ Hydrindantin + Decarboxylated amino acid + Carbon dioxide + Ammonia

Hydrindantin + Ninhydrin + Ammonia ⟶ Purple coloured product + water

Reagents

1. 10% Tri chloro acetic acid (TCA),
2. Ninhydrin reagent
3. Stock Standard Tyrosine: Dissolved 50 mg of tyrosine in 50 ml of distilled water.
4. Working standard Tyrosine: 10 ml of the stock standard was made upto 100 ml. 1 ml of the sample gives a concentration of 100 µg.

Procedure

10% homogenates of gill and muscle and 5% homogenate of liver followed by 0.5% homogenate of heart and 1% homogenate of kidney tissues were prepared in 10% TCA. The tissues were then centrifuged at 1000g for 15 minutes. The supernatant was taken for the assay. To 0.5 ml of the supernatant, 2.0 ml of ninhydrin reagent was added. The tubes were kept in a boiling water bath for 5 minutes. It was then cooled immediately. After suitable cooling the volume was made up to 10 ml with distilled water and the absorbance was read at 570 nm in a spectrophotometer using a blank. The blank consists of 0.5 ml of 10% TCA and 2 ml of ninhydrin reagent and received the same treatment as that of sample. The amino acid content was expressed as µmoles of tyrosine equivalent/g wet wt of tissue.

h. Estimation of acid phosphatase

Principle

The method used was that of King and Armstrong (1934); King *et al.*, (1937, 1942) in which disodium phenyl phosphate gets hydrolyzed with the liberation of phenol and inorganic phosphate. The liberated phenol was measured at 700 nm with Folin-Ciocalteau reagent.

Reagents

1. Citrate buffer of 0.1 M, pH 5

   A:Citric acid - 21.01 g was dissolved in 1000 ml of distilled water

   B:Sodium citrate - 29.41 g was dissolved in 1000 ml of distilled water

   Mixed 20.5 ml of A and 29.5 ml of B. The pH was adjusted to 5. It was then made up to 100 ml with distilled water.

2. Disodium phenyl phosphate, 100 mmol/l. Dissolved 2.18 g of disodium phenyl phosphate in distilled water. It was then heated to boil, cooled and was then made to a litre. To this 1.0 ml of chloroform was added. It was then stored in the refrigerator.
3. Buffer – substrate: This was prepared by mixing equal volume of the above two solutions. This has a pH of 5.0
4. Folin Ciocalteau reagent: Mixed 1.0 ml of reagent with 2.0 ml of water (1:2 ratio).
5. Sodium carbonate (Na_2CO_3) solution 15%: Dissolved 15 g of anhydrous sodium carbonate in 100 ml of water.
6. Standard phenol solution, 1 g/L: Dissolved 1 g of pure crystalline phenol in 100 mmol/L HCl.
7. Working standard solution: Diluted 10 ml of stock standard to 100 ml with water. This contains 100 µg phenol/ml.

Procedure

10% homogenate of gill and muscle; 2% homogenate of liver and 1% homogenate of kidney and heart tissues were prepared in 0.33 M ice cold sucrose solution. The homogenates were then centrifuged at 1000g for 15 minutes. The supernatant was taken for the assay. Pipetted 4.0 ml of the buffer substrate into a test tube. It was then incubated at 37^0C for 5 minutes. To this 0.2 ml of the sample was added. The tube was incubated further for exactly 60 min. After incubation, the tubes were removed. To this immediately 1.8 ml of diluted phenol reagent was

added. At the same time a control was set up containing 4.0 ml buffer substrate and 0.2 ml sample to which 1.8 ml phenol reagent was added immediately. All the tubes were then mixed well and centrifuged. To 4.0 ml of the supernatant 2.0 ml of sodium carbonate solution was added. Taken 4.0 ml of working standard solution and for blank 3.2 ml of water was taken. Into this 0.8 ml of phenol reagent was added. Then added 2 ml of sodium carbonate. All the tubes were incubated at 37^0C for 15 min. The colour obtained was then read at 700 nm. The enzyme activity were expressed as units/L for serum and units/protein for tissues.

7.3C Results

Table 7.3.1 Effect of exposure to different concentrations of ethanol for 7 days on serum parameters of *O. mossambicus*.

Serum Parameters Investigator	Control	Concentrations of ethanol 0.65g/l	1.3g/l	2.6g/l
Albumin (g/dl)	0.525± 0.01870	0.143± 0.0102	0.112± 0.0097	0.034± 0.0080
Urea (mg/dl)	9.408± 0.2521	8.517± 0.3340	7.228± 0.1579	6.357± 0.2277
Uric acid (mg/dl)	0.345± 0.0302	0.475± 0.0372	0.410± 0.0079	1.417± 0.1443
Creatinine (mg/dl)	0.142± 0.0331	0.233± 0.0308	0.340± 0.0323	0.448± 0.0331
Ammonia (µmol/L)	6.164± 0.0060	6.281± 0.0063	6.600± 0.0048	6.614± 0.0036

Average of six values in each group ± SD of six observations.

Table 7.3.2 Effect of exposure to different concentrations of ethanol for 21 days on serum parameters of *O. mossambicus*.

Serum Parameters Investigators	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Albumin (g/dl)	0.550± 0.0089	0.335± 0.0105	0.228± 0.0147	0.083± 0.0095
Urea (mg/dl)	11.09± 0.6509	5.723± 0.3677	5.075± 0.0418	4.515± 0.3440
Uric acid (mg/dl)	0.452± 0.0319	0.543± 0.0301	0.623± 0.0230	0.828± 0.0256
Creatinine (mg/dl)	0.252± 0.0319	0.353± 0.0308	0.443± 0.0288	0.538± 0.0286
Ammonia (μmol/L)	6.165± 0.0056	6.185± 0.0068	6.067± 0.0060	5.955± 0.0057

Average of six values in each group ± SD of six observations.

Table 7.3.3 Effect of exposure to different concentrations of ethanol for 7 days on total protein content present in different tissues of *O. mossambicus*.

Tissues Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Gills	14.42± 1.5494	9.620± 1.0296	6.995± 0.2790	4.952± 0.5044
Muscle	21.44± 0.6362	12.93± 0.5600	10.24± 0.5162	5.357± 0.9034
Liver	65.93± 3.3305	95.10± 2.2442	72.73± 1.9189	32.41± 2.1684
Kidney	26.56± 0.6986	31.32± 0.6073	41.42± 0.8496	15.50± 1.5136
Heart	46.19± 3.0907	41.44± 0.4674	35.87± 2.7202	22.75± 3.5006

Values are expressed as mg/g wet weight of tissue

Average of six values in each group ± SD of six observations.

Table 7.3.4 Effect of exposure to different concentrations of ethanol for 21 days on total protein content present in different tissues of *O. mossambicus*.

Tissues Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Gills	29.42± 0.6842	23.17± 0.9685	16.68± 1.0640	11.33± 0.9222
Muscle	32.39± 0.3807	16.93± 1.4809	13.04± 0.5430	10.37± 0.4971
Liver	103.6± 2.8892	99.08± 2.6439	95.59± 1.0512	74.23± 1.1872
Kidney	98.49± 1.2915	91.21± 0.4721	88.07± 1.1676	83.91± 1.1540
Heart	94.84± 2.9989	81.46± 0.9970	73.83± 2.6061	54.33± 3.4000

Values are expressed as mg/g wet weight of tissue.

Average of six values in each group ± SD of six observations.

Table 7.3.5 Effect of exposure to different concentrations of ethanol for 7 days on total free amino acid content present in different tissues of *O. mossambicus*.

Tissues Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Gills	3.145± 0.0423	3.377± 0.0308	3.450± 0.0283	3.470± 0.0447
Muscle	3.048± 0.0213	3.142± 0.0184	3.195± 0.0105	3.245± 0.0105
Liver	3.757± 0.0301	4.150± 0.3391	3.913± 0.0561	3.688± 0.0331
kidney	3.748± 0.0147	3.803± 0.0163	4.213± 0.0476	4.428± 0.0331
Heart	3.822± 0.0232	3.958± 0.0214	4.077± 0.0509	4.232± 0.0376

Values are expressed as μ moles of tyrosine equivalent/g wet weight of tissue.

Average of six values in each group ± SD of six observations.

Table 7.3.6 Effect of exposure to different concentrations of ethanol for 21 days on total free amino acid content present in different tissues of *O. mossambicus.*

Tissues Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Gills	3.468± 0.0147	3.545± 0.0187	3.567± 0.0250	3.677± 0.0550
Muscle	3.170± 0.0089	3.248± 0.0117	3.370± 0.0141	3.467± 0.0163
Liver	4.232± 0.0231	4.495± 0.0524	4.303± 0.0273	4.178± 0.0231
Kidney	3.942± 0.0286	4.335± 0.0485	4.460± 0.0237	4.740± 0.0228
Heart	4.045± 0.0259	4.218± 0.0147	4.325± 0.0152	4.435± 0.0288

Values are expressed as μ moles of tyrosine equivalent/g wet weight of tissue

Average of six values in each group ± SD of six observations.

Table 7.3.7 Effect of exposure to different concentrations of ethanol for 7 days on acid phosphatase activity present in serum and different tissues of *O. mossambicus.*

Sample Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Serum	16.24± 1.1452	31.27± 0.9680	34.76± 0.2908	42.77± 3.0800
Gills	0.592± 0.0110	0.631± 0.0153	0.785± 0.0460	1.093± 0.2341
Muscle	0.167± 0.0272	0.356± 0.0369	0.444± 0.0082	0.723± 0.0474
Liver	0.702± 0.0053	0.815± 0.0115	0.922± 0.0152	1.075± 0.0401
Heart	0.256± 0.0426	0.480± 0.0193	0.660± 0.0379	1.052± 0.1041
Kidney	0.667± 0.0085	0.733± 0.0188	0.842± 0.0130	0.908± 0.0080

Values are expressed as μ moles/mg protein/h in tissues and Units/L in serum.

Average of six values in each group ± SD of six observations.

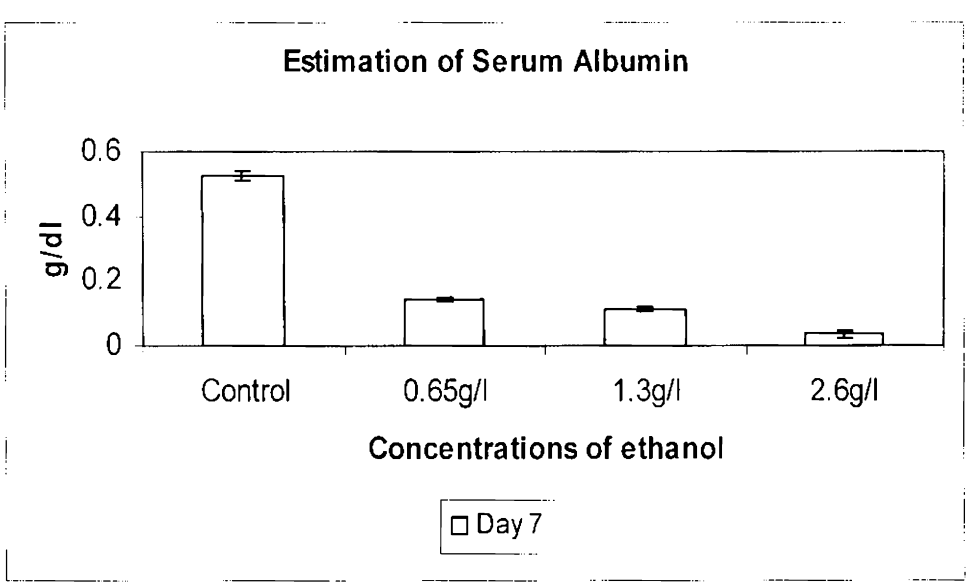
Table 7.3.8 Effect of exposure to different concentrations of ethanol for 21 days on acid phosphatase activity present in serum and different tissues of *O. mossambicus*.

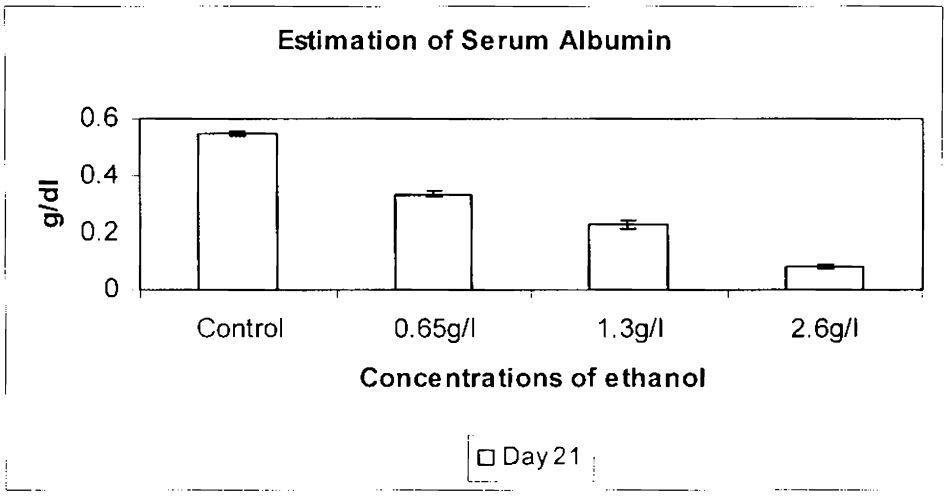
Sample Analyzed	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Serum	19.86± 0.7704	23.68± 0.9503	26.89± 0.8965	37.60± 1.1538
Gills	0.379± 0.0063	0.483± 0.0121	0.570± 0.0083	0.622± 0.0117
Muscle	0.197± 0.0044	0.217± 0.0059	0.244± 0.0044	0.267± 0.0019
Liver	0.715± 0.0026	0.807± 0.0102	1.040± 0.0564	2.245± 0.1633
Heart	0.371± 0.0065	0.459± 0.0111	0.612± 0.0576	0.780± 0.0195
Kidney	0.750± 0.0054	0.850± 0.0114	0.923± 0.0085	1.208± 0.0092

Values are expressed as μ moles/mg protein/h in tissues and Units/L in serum.

Average of six values in each group ± SD of six observations.

Figure 7.3.9 Levels of albumin in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant decrease ($P<0.001$) in serum albumin level was noted in *O. mossambicus* when treated with various sub lethal concentrations of ethanol, with respect to control during 7 and 21 days of exposure period (Figure 7.3.9). ANOVA has been carried out to ascertain the statement and the table is shown below (Table 7.3.9a).

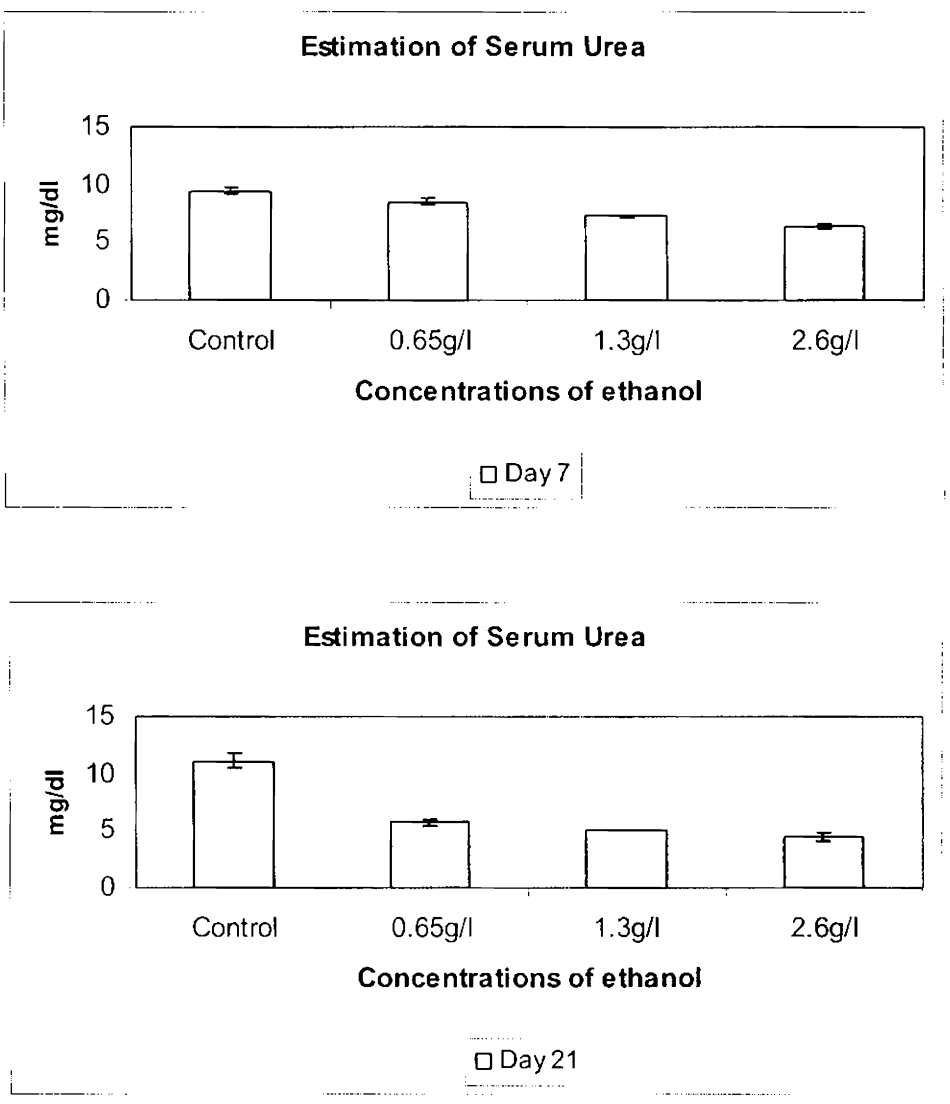
Table 7.3.9a ANOVA table for serum albumin

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.109	1	0.109	789.205	0.000
Between Concentrations	1.510	3	0.503	3629.632	0.000
Days of Exposure × Concentrations	0.051	3	0.017	121.777	0.000
Error	0.006	40	0.000		
Total	1.676	47			

df- degrees of freedom

Two Factor ANOVA table revealed that serum albumin levels varied significantly between days ($P<0.001$). Between concentrations there was a significant difference ($P<0.001$). Also while taking into consideration the effects of both the days as well as concentrations, (Interaction) significant difference ($P<0.001$) was observed.

Figure 7.3.10 Levels of urea in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Serum urea level was found to be significantly decreased ($P<0.001$) in all the three sub lethal concentrations of ethanol (Figure 7.3.10) with respect to control in *O. mossambicus* in both durations of exposure. To validate this ANOVA was carried out and the results obtained are depicted below (Table 7.3.10a).

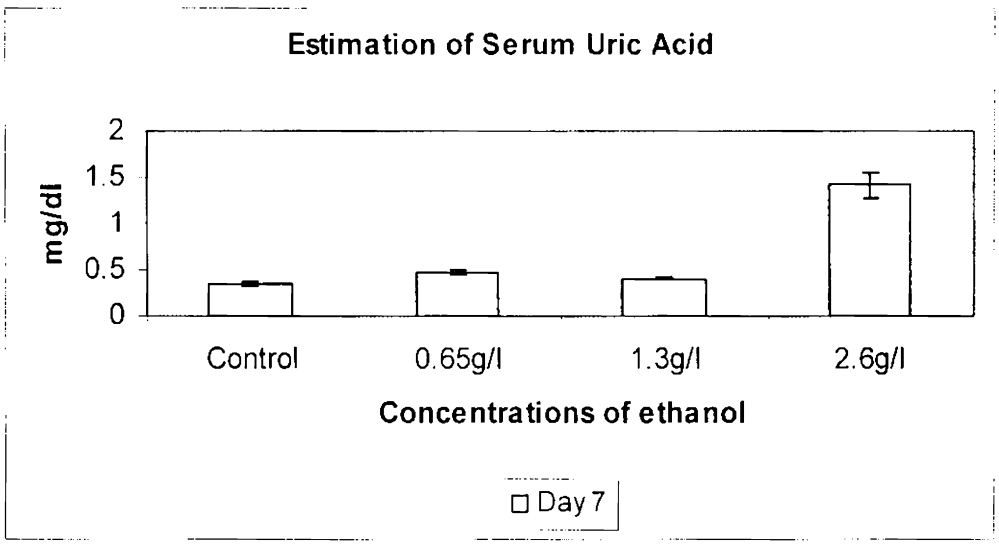
Table 7.3.10a ANOVA table for serum urea

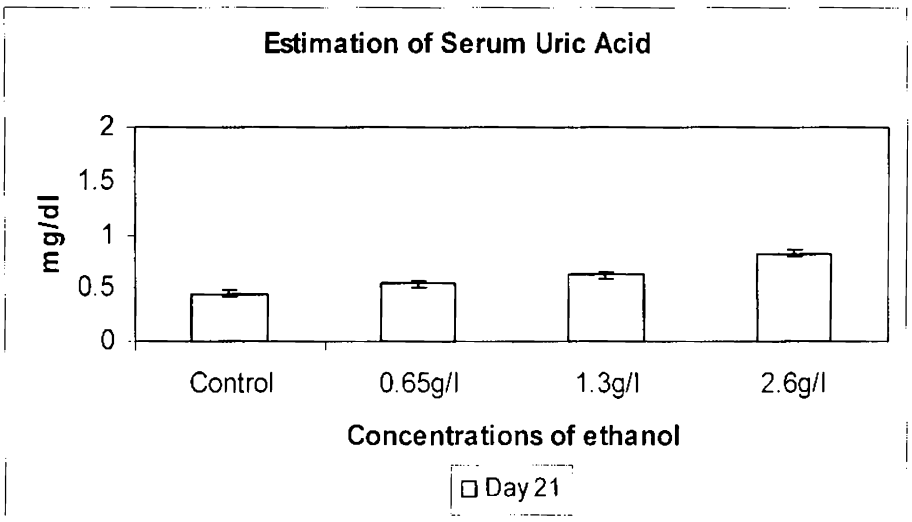
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	19.546	1	19.546	167.986	0.000
Between Concentrations	162.168	3	54.056	464.583	0.000
Days of Exposure × Concentration	36.449	3	12.150	104.420	0.000
Error	4.654	40	0.116		
Total	222.817	47			

df- degrees of freedom

Studies done by using Two Factor ANOVA indicated that serum urea levels varied significantly between days ($P<0.001$). There was a significant difference ($P<0.001$) between concentrations. While comparing both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was noted.

Figure 7.3.11 Levels of uric acid in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





From the graph (Figure 7.3.11) it can be concluded that serum uric acid levels varied significantly ($P<0.01$) in *O. mossambicus* during immediate and prolonged exposure periods. Employing ANOVA justifies the above statement (Table 7.3.11a).

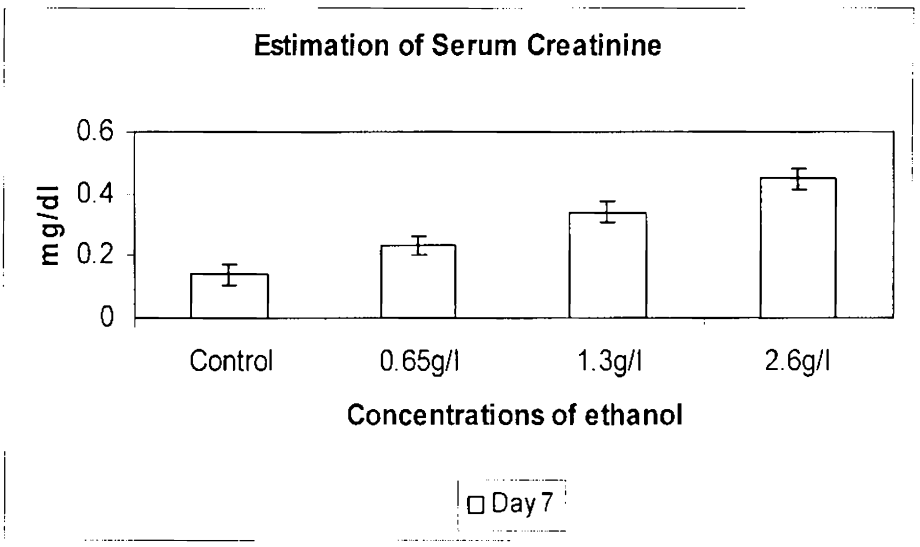
Table 7.3.11a ANOVA table for serum uric acid

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.030	1	0.030	9.114	0.004
Between Concentrations	3.884	3	1.295	393.983	0.000
Days of Exposure × Concentration	1.193	3	0.398	121.017	0.000
Error	0.131	40	0.003		
Total	5.238	47			

df- degrees of freedom

By using Two Factor ANOVA it was noted that serum uric acid level varied significantly between days ($P<0.01$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 7.3.12 Levels of creatinine in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



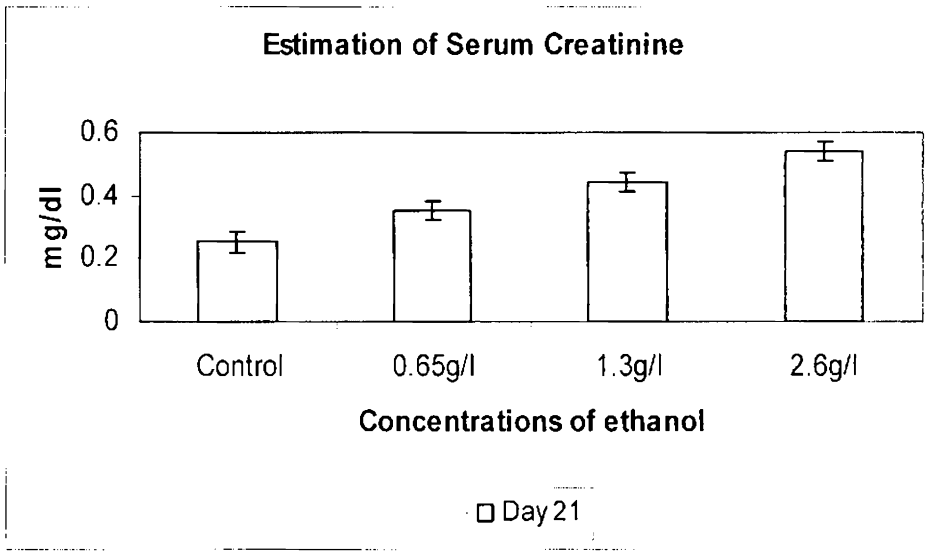


Figure 7.3.12 depicts marked alterations in the serum creatinine levels in *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure period. Analysis carried out by applying ANOVA supports the above statement (Table 7.3.12a).

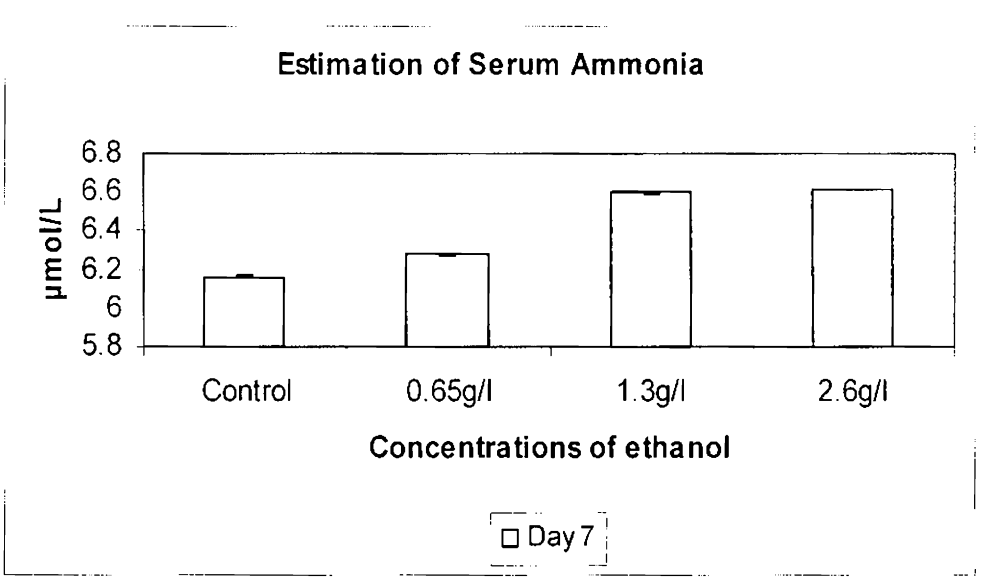
Table 7.3.12a ANOVA table for serum creatinine

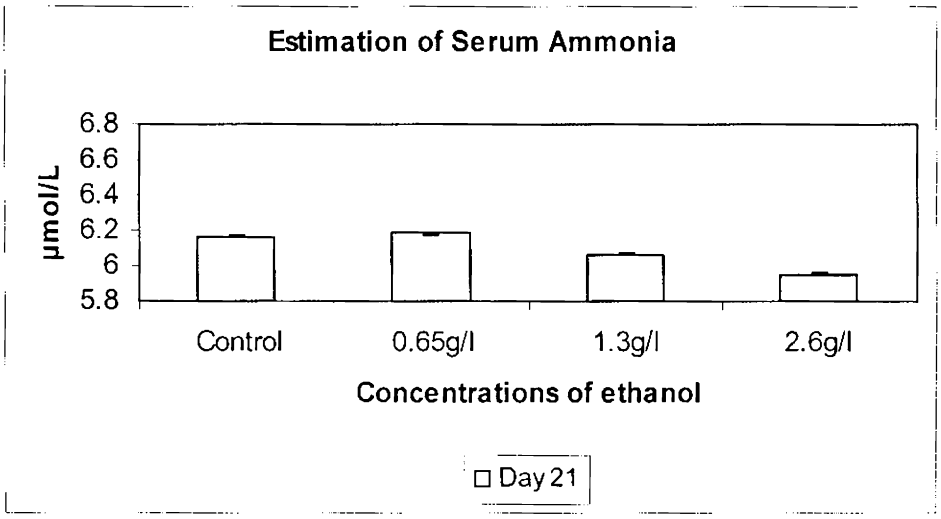
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.134	1	0.134	138.091	0.000
Between Concentrations	0.586	3	0.195	200.739	0.000
Days of Exposure × Concentration	0.001	3	0.000	0.488	0.693
Error	0.039	40	0.001		
Total	0.760	47			

of- degrees of freedom

Analysis by Two Factor ANOVA indicated that serum creatinine levels varied significantly ($P<0.001$) between days. Between concentrations a marked significant difference ($P<0.001$) was noted. Considering the interaction effects of both the days as well as concentrations, (Interaction) no significant difference was seen.

Figure 7.3.13 Levels of ammonia in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





O. mossambicus exposed to varying sub lethal concentration of ethanol exhibited marked significant ($P<0.001$) (Figure 7.3.13) changes in the serum ammonia value. The ANOVA table mentioned below (Table 7.3.13a) justifies this conclusion.

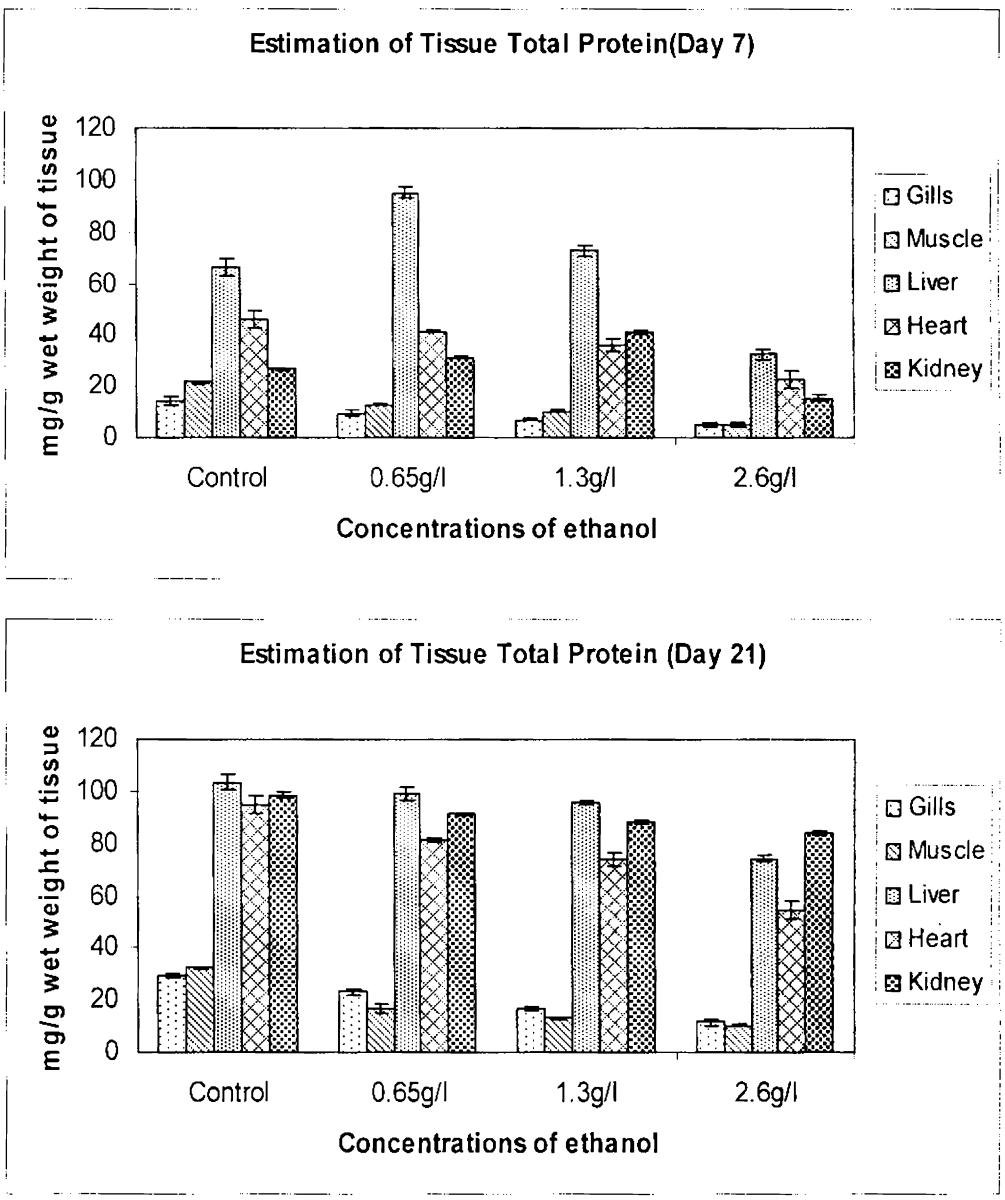
Table 7.3.13a ANOVA table for serum ammonia

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	1.243	1	1.243	38790.703	0.000
Between Concentrations	0.189	3	0.063	1968.283	0.000
Days of Exposure × Concentration	0.940	3	0.313	9782.444	0.000
Error	0.001	40	3.20E-005		
Total	2.373	47			

df- degrees of freedom

Statistical analysis done by using Two Factor ANOVA revealed that serum ammonia levels varied significantly between days ($P<0.001$). It was noted that between concentrations significant difference was ($P<0.001$). It was concluded that by taking into consideration both the days as well as concentrations (Interaction) significant difference ($P<0.001$) was observed.

Figure 7.3.14 Levels of total protein content present in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Marked significant ($P<0.001$) (Figure 7.3.14) decrease in total protein content was observed in the tissues (viz. gills, muscle, liver, heart and kidney) of *O. mossambicus* in all the three sub lethal dosage groups, with respect to control group on both 7 and 21 days. In order to substantiate the above statement ANOVA was taken into account and the results are mentioned below (Table 7.3.14a).

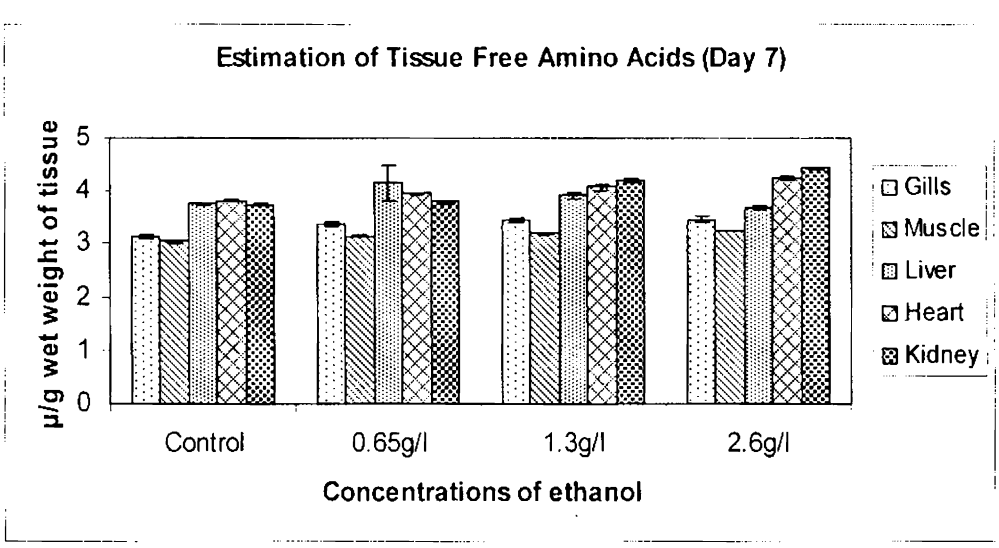
Table 7.3.14a ANOVA table for tissue total protein

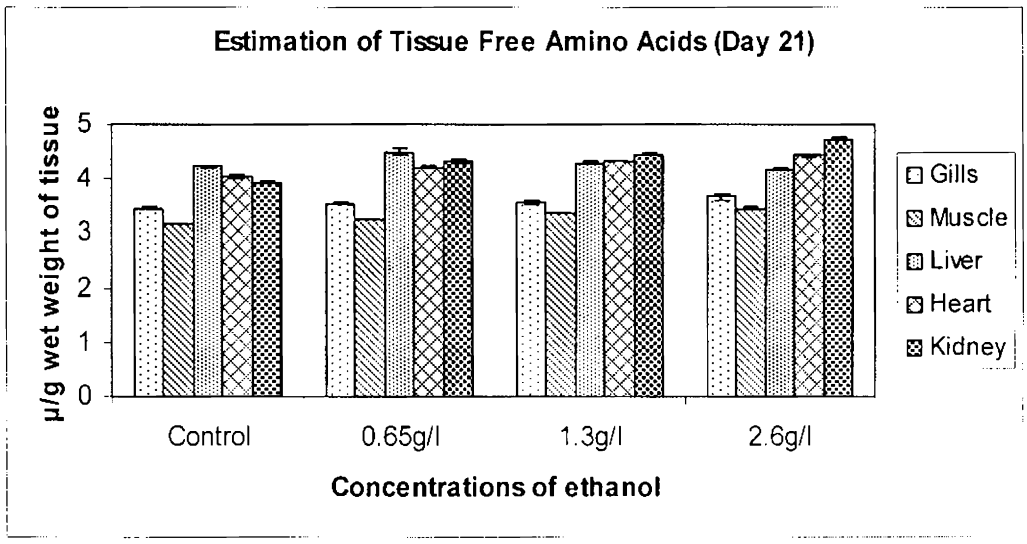
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	50247.075	1	50247.075	354.296	0.000
Between Concentrations	16714.808	3	5571.603	34.427	0.000
Between Tissues	161234.732	4	40308.683	249.067	0.000
Error	37384.751	231	161.839		
Total	265581.365	239			

df- degrees of freedom

Studies done using Three Factor ANOVA table exhibited that between days of exposure significant difference ($P<0.001$) was obtained. When taken into consideration the effect between concentrations significant difference ($P<0.001$) was noted. It was also observed that between tissues significant difference of ($P<0.001$) was obtained.

Figure 7.3.15 Levels of total free amino acids present in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Significant increase (P<0.001) in free amino acid level (Figure 7.3.15) was observed in the tissues of *O. mossambicus* exposed to all the three sub lethal concentrations of ethanol with respect to control group. To validate this ANOVA was carried out and the results are depicted below (Table 7.3.15a).

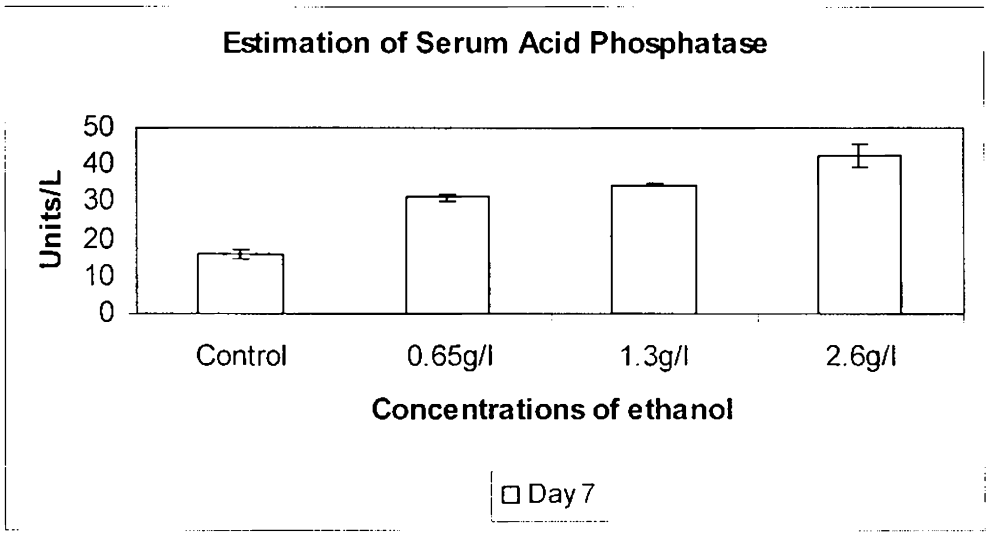
Table 7.3.15a ANOVA table for tissue free amino acids

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	5.125	1	5.125	245.632	0.000
Between Concentrations	2.368	3	0.789	37.839	0.000
Between Tissues	40.519	4	10.130	485.535	0.000
Error	4.819	231	0.021		
Total	52.831	239			

df- degrees of freedom

Statistical analysis done using Three Factor ANOVA table revealed that between days of exposure, between concentrations and also between tissues significant difference (P<0.001) was obtained.

Figure 7.3.16 Levels of acid phosphatase activity in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



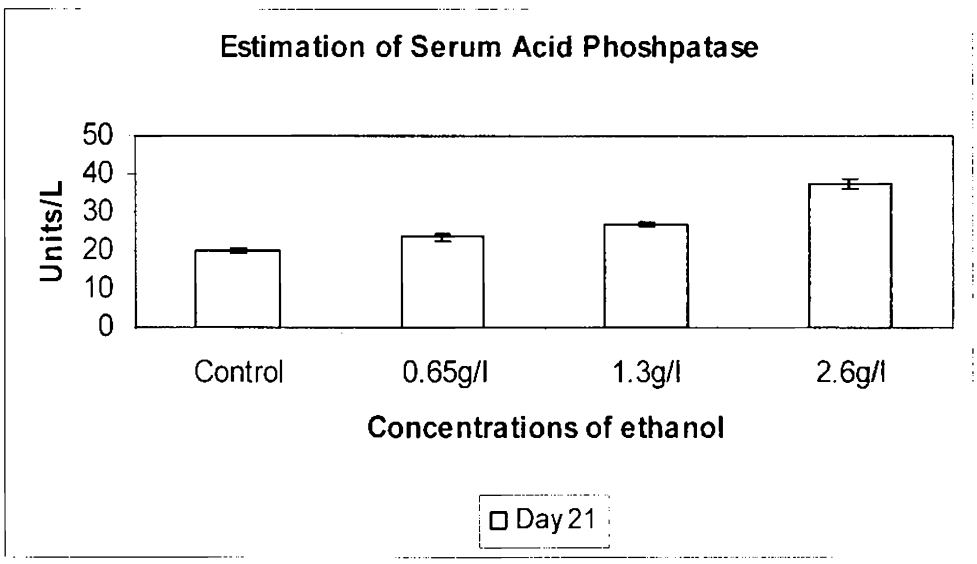


Figure 7.3.16 depicts significant increase ($P<0.001$) in the serum acid phosphatase levels in *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure period. Analysis carried out by applying ANOVA supports the above statement (Table 7.3.16a).

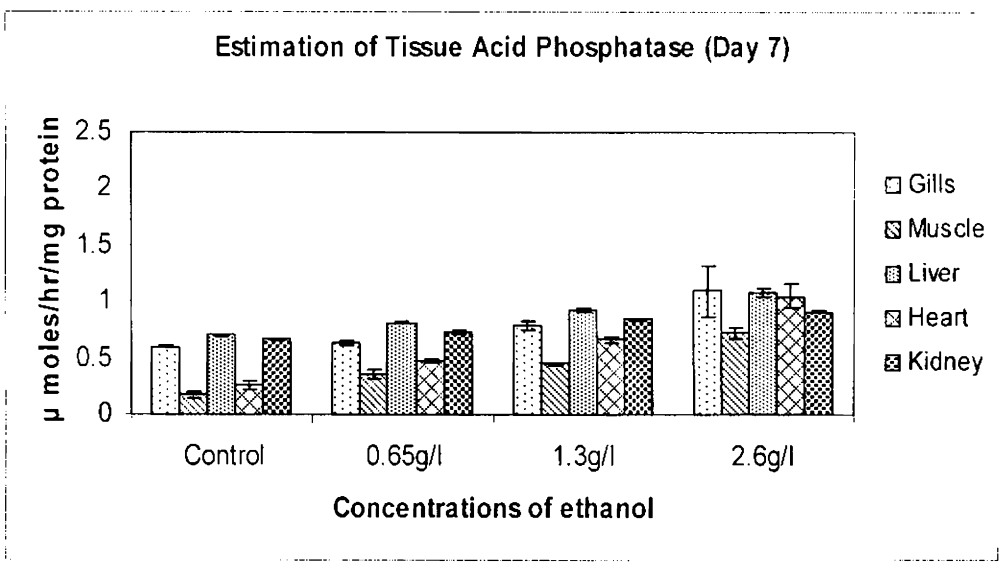
Table 7.3.16a ANOVA table for serum acid phosphatase

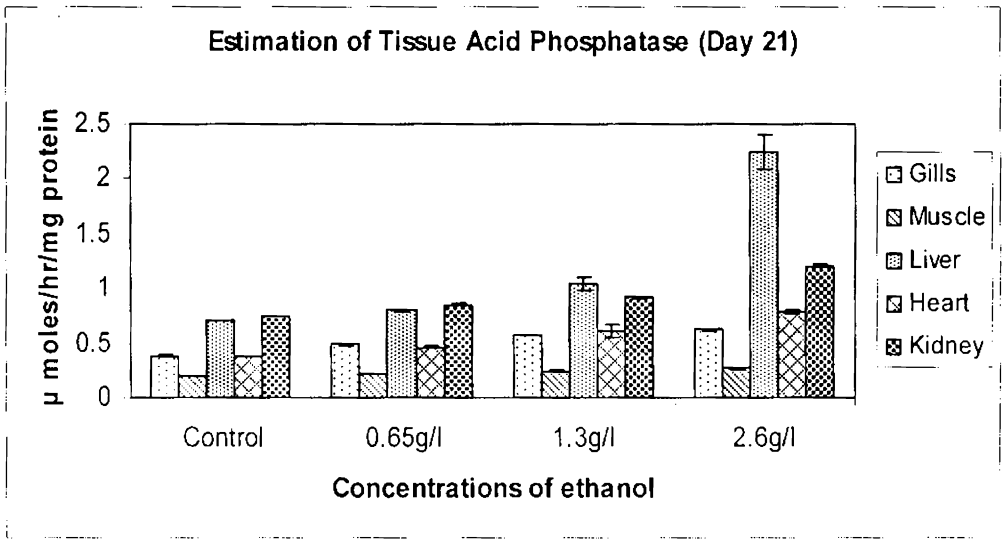
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	216.878	1	216.878	112.293	0.000
Between Concentrations	3006.931	3	1002.310	518.966	0.000
Days of Exposure × Concentration	260.846	3	86.949	45.019	0.000
Error	77.254	40	1.931		
Total	3561.909	47			

df- degrees of freedom

Two Factor ANOVA table revealed that in the case of serum alkaline phosphatase levels, between days, significant difference ($P<0.001$) was observed. Between concentrations there was a significant difference ($P<0.001$). When taken into consideration the effects of both days as well as concentrations, (Interaction) significant difference ($P<0.001$) was noted.

Figure 7.3.17 Levels of acid phosphatase activity in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Relevant changes were noted in the acid phosphatase values (Figure 7.3.17) in the tissues ((viz. gills, muscle, liver, heart and kidney) of *O. mossambicus* exposed to 7 and 21 days. Analysis using ANOVA authenticate this (Table 7.3.17a).

Table 7.3.17a ANOVA table for tissue acid phosphatase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.007	1	0.007	0.161	0.688
Between Concentrations	9.073	3	3.024	73.752	0.000
Between Tissues	14.294	4	3.573	87.139	0.000
Error	9.432	230	0.041		
Total	32.806	238			

df- degrees of freedom

Three Factor ANOVA table stated that there were no significant difference when days of exposure were taken into consideration. But when considering the effect between concentrations and also between tissues significant difference ($P<0.001$) was obtained.

Subsequent comparisons by multiple comparison test using Tukey followed by Dunnett's method is shown below (Table 7.3.18 and 7.3.19)

Table 7.3.18 Multiple Comparison Test (Concentrations)

	Groups	Serum Albumin	Serum Urea	Serum Uric acid	Serum Creatinine	Serum Ammonia	Tissue Total Protein	Tissue Free Amino Acids	Serum ACP	Tissue ACP
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.402[d]	0.000[a]	0.000[a]	0.016[c]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.002[b]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

Table 7.3.19 Multiple Comparison Test (Tissues)

	Tissues	Tissue Total Protein	Tissue Free Amino Acids	Tissue ACP
Tukey	Gills Vs Muscle	0.998[d]	0.000[a]	0.000[a]
	Gills Vs Liver	0.000[a]	0.000[a]	0.000[a]
	Gills Vs Heart	0.000[a]	0.000[a]	0.590[d]
	Gills Vs Kidney	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Liver	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Heart	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Kidney	0.000[a]	0.000[a]	0.000[a]
	Liver Vs Heart	0.000[a]	0.452[d]	0.000[a]
	Liver Vs Kidney	0.000[a]	0.000[a]	0.000[a]
	Heart Vs Kidney	0.728[d]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001 and not significant at d.

A subsequent pair wise comparison between various concentrations with respect to control using Dunnett's method revealed that parameters such as serum albumin, urea, uric acid, creatinine, ammonia and acid phosphatase exhibited significant difference (P<0.001). In the case of tissue total protein, fishes exposed to ethanol of 0.65 g/l concentration exhibited no significant difference when being compared with control, whereas 1.3 g/l exhibited significant difference (P<0.01) and 2.6 g/l exhibited significant difference (P<0.001) when compared with control. In the case of tissue free amino acids significant difference (P<0.001) was obtained in all the three concentrations when compared with control. When taken into

consideration tissue acid phosphatase of fishes exposed to ethanol of concentration 0.65 g/l, significant difference ($P<0.05$) was obtained and in the case of fishes exposed to 1.3 g/l and 2.6 g/l ethanol, significant difference ($P<0.001$) was noted when being compared with control.

Subsequent pair wise comparison between various tissues were carried out using Tukey which indicated that in the case of tissue total protein muscle tissue when compared with gills exhibited no significant difference (NS), whereas tissues such as liver, heart and kidney when compared with gill tissue exhibited significant difference($P<0.001$). Also when liver, heart and kidney tissue were being compared with muscle, significant difference ($P<0.001$) was noted. Similarly when heart and kidney tissue were compared with liver significant difference ($P<0.001$) was noted. Also kidney tissue when compared with heart showed no significant difference. In the case of tissue free amino acids muscle, liver, heart and kidney when being compared with gills showed significant difference ($P<0.001$). Also liver, heart and kidney tissue when being compared with muscle exhibited significant difference ($P<0.001$). Simultaneously no significant difference was observed when heart tissue was compared with liver. It was concluded by stating that liver and heart when compared with kidney exhibited significant difference ($P<0.001$). In the case of tissue ACP, muscle, liver and kidney tissues when compared with gills exhibited significant difference ($P<0.001$) whereas heart tissues when compared with gills showed no significant difference. Liver, heart and kidney tissues when compared with muscle exhibited significant difference ($P<0.001$). Also heart and kidney tissues when compared with liver exhibited significant difference ($P<0.001$). Similarly kidney tissues when compared with heart too exhibited significant difference ($P<0.001$).

7.3D Discussion

The reports available from early studies indicate that xenobiotic metabolism in fishes takes place at a slower rate than in mammalian systems (Wheelock *et al.*, 2005). It has also been made clear by stating that the metabolism of xenobiotics in fish seems to be different from that in mammals (Bello *et al.*, 2001; Fulton and Key,

2001; Wheelock *et al.*, 2005; Whyte *et al.*, 2000). Nitrogenous indices evaluated in this study are referred to as useful parameters that indicate impairment in the functional capacity of organs. Urea is the principal end product of protein catabolism most in terrestrial animals. Mayer *et al.* (1992) have stated biomarkers as alterations occurring at physiological, biochemical and histopathological levels as a result of exposure to environmental pollution. Enzyme activities may change by physiological conditions and other environmental factors (Lopes *et al.*, 2001). The role of blood enzymes employed in monitoring and detecting stress or disease has led to wide - spread usage of these as biochemical indicators to trace environmental pollutants (Adham *et al.*, 1997, 1999; William, 1997). Biomarkers such as LDH, AST, CE and ACP have been employed for diagnosing liver, muscle and gill damages caused by pollutants in fish (Neff, 1985). Serum biochemistry has therefore been used as a sensitive tool to assess the toxic impact of contaminants. These same biomarkers can also be used to determine the baseline health and physiology of aquatic organisms. The present results clearly revealed the harmful impact of ethanol on protein metabolism of aquatic organisms. Urea occurs in nature as the major nitrogen containing end product of protein metabolism in vertebrates. In the case of teleosts urea is normally synthesized via uricolysis pathway in which uric acid is converted into urea and glyoxylate via uricase, allantoinase and allantoicase. Uric acid is a purine, which is produced from the breakdown of body cells. Creatinine, a nitrogenous waste product, is synthesised in the body at a fairly constant rate from creatine. In fish, urea can be excreted or reabsorbed at two main locations, the gill and the kidney. Knowledge regarding the composition of blood and function of its components seems to be a fundamental necessity to understand the normal and pathological physiology of animal.

Hypoalbuminemia seems to be a very common feature in the case of chronic alcoholic liver disease (Annoni *et al.*, 1991; Das *et al.*, 2003). Hypoalbuminemia is frequently found in the end stage of liver disease. Decreased serum albumin is an important indicator of end stage liver disease. Albumin was found to decrease in many other conditions in addition to cirrhosis. Protein losing enteropathy and malabsorption conditions such as chronic pancreatitis or small intestinal malabsorptive diseases such as sprue resulted in hypoalbuminemia. In the present

study a significant decrease (P<0.001) (Fig 7.3.9) in serum albumin level was observed when *O. mossambicus* was exposed to ethanol for 7 and 21 days. Acute exposure to alcohol depressed albumin. This in turn reflects disturbances in liver function integrity, as found in the experimental groups. A decrease in serum albumin was observed when male and female rats were exposed to benzene for 10 days by Rao *et al.*, (1994). Common features of chronic alcoholic liver disease include progressive hypoalbuminemia (Annoni *et al.*, 1990; Rothschild *et al.*, 1983).

A significant decrease (P<0.001) (Fig 7.3.10) in serum urea level was observed in *O. mossambicus* when subjected to varying sub lethal concentrations of ethanol for 7 and 21 days. Decrease in serum urea level on exposure to ethanol was strongly supported by Das and Vasudevan. (2005) who too observed similar changes in the serum of alcoholics with alcoholic liver disease. Urea concentrations were often reduced because alcohol inhibits enzymes in the urea cycle.

Uric acid is the end product of the catabolism of tissue nucleic acid, i.e. metabolism of purines and pyrimidine bases. In the present study an increase in serum uric acid was observed when *O. mossambicus* was subjected to immediate and prolonged exposure to ethanol (Fig 7.3.11). However, if too much uric acid is being produced or if kidneys are not able to remove it from blood normally, the level of uric acid in blood normally increases. Ashour *et al.* (2007) supported the above statement by reporting elevated uric acid (p<0.05) in rats when exposed to sub lethal concentration of lead acetate. Increase in seum uric acid level was reported by Das and Vasudevan. (2005) who observed similar changes in the serum of alcoholics with alcoholic liver disease. The significant increase in serum uric acid and creatinine values refer to kidney failure, increased muscular tissue catabolism, decreased urinary clearance by the kidney, increased synthesis or decreased degradation of these compounds etc as mentioned by Fruton and Simmonds (1958); Emmerson (1973). Increase in serum uric acid and serum creatinine values was found in the blood chemistry of Nile tilapia *O. niloticus* (Linnaeus, 1757) under the impact of water pollution.

A significant increase (P<0.001) (Fig 7.3.12) in the serum creatinine values were observed when *O. mossambicus* was subjected to different sub lethal

concentrations of ethanol for 7 and 21 days. The increase in the serum creatinine value can be due to harmful effects on kidney tissues brought about by ethanol. Another possible reason for the increase in serum creatinine values mainly arises due to glomerular insufficiency, increased muscle tissue catabolism or impairment of the carbohydrate metabolism. This theory has been reported by Murray *et al.* (1990) who also recorded similar increase in serum creatinine values in fishes caught from heavily and moderately polluted areas. Abdelmeguid *et al.* (2002); Maria *et al.* (2004) supports the present data of increase in serum creatinine values. Studies conducted by Soufy *et al.* (2007) on monosex Tilapia on chronic exposure to carbofuran pesticide is also in agreement with the present observation.

Ethanol is regarded primarily as a neurotoxin and hepatotoxin. A significant increase ($P<0.001$) (Fig 7.3.13) in serum ammonia was noted when *O. mossambicus* was subjected to exposure to ethanol for 7 days. The increase in the serum ammonia level could be due to increased protein catabolism. Similar findings were made by Sokolovic (2007) when rats were treated with ethanol. Another possible reason for the increase in serum ammonia level could be the pathological changes occurring in the gill tissues which results in respiratory problems, leading to stress. Fishes which are unable to excrete this metabolic waste product, accumulate it resulting in the rise of serum ammonia levels by bringing about damage to the organs. A significant increase in serum ammonia level was observed in fishes exposed to ethanol of concentration 0.65g/l followed by a decrease at 1.3 g/l and 2.6 g/l ethanol for a period of 21 days. The decrease in serum ammonia level was cited by Rolf *et al.* (1997) in cirrhotic rats subjected to chronic toxicity studies. Most teleost fishes are ammoniotelic, excreting ammonia as the major nitrogenous waste product by diffusion across the gills into the aqueous environment (Campbell 1991; Wood, 1993; Wilkie, 1997; Ip *et al.* 2001b). Under normal conditions ammonia is detoxified in the liver by conversion to urea and glutamine. It can also be inferred that cirrhosis brings about an elevation in serum ammonia levels. The findings stated by David (2008) supports this statement.

Proteins have been mainly involved in the architecture of the cell. It is said to be the chief source of nitrogenous metabolism. Tissue proteins of aquatic animals

under toxic stress are known to play a pivotal role in the activation of compensatory mechanisms (Venkataramana and Radhakrishnaiah, 1987). In the present study an increase in tissue total protein content was observed exposed to 0.65 g/l and 1.3 g/l ethanol, but those exposed to 2.6 g/l ethanol recorded a decrease in total protein content in the liver and kidney tissues of *O. mossambicus* when exposed to 7 days (Fig 7.3.14). The increase in tissue total protein level in liver and kidney tissues was mainly due to ethanol which brought about stress to the fish. In order to overcome this toxicant stress, increased biosynthesis of proteins might have taken place. The same findings have been cited by Sornarej *et al.* (1995) who too stated that the increase in the protein content could be due to the enhanced synthesis of proteins to meet the enhanced demand for energy under stress, or to compensate the tissue necrosis which arises when *O. mossambicus* were exposed to varying concentrations of ethanol. Similar findings have been made by Shobha *et al.* (2001) in the liver and kidney tissues of *O. mossambicus* when exposed to 96 hours to study the effect of arsenite. The decrease on exposure to ethanol at 2.6 g/l ethanol was supported by Vijayamohanan and Achutan (2000) who also observed a decrease in the protein level in the tissues of the fresh water fishes *O. mossambicus* and *E. maculates* exposed for seven days to three sub lethal concentrations of titanium dioxide industrial effluent. Simultaneously all the other tissues such as gills, muscle and heart tissues exhibited a decrease in the total protein content when exposed to 7 days. This decrease in the total protein content explains that the protein was used as an alternative source of energy, due to high energy demand brought about by ethanol intoxication. Observations of Hori *et al.* (2006) who observed similar trend in fish tissues when subjected to phenol intoxication support this statement. A significant decrease ($P<0.001$) (Table 7.3.14a) in tissue total protein was observed when fish was exposed to ethanol for 21 days (Fig 7.3.14). This could be due to the decrease in protein content in tissues, resulting in decreased biosynthesis of proteins. It can also be inferred that proteins get degraded into free amino acids which are used for the different metabolic activities arising during ethanol induced stress condition. Anees (1974) is also of the same view. Another possible reason could be that ethanol

intoxication leads to enhanced degradation of protein and simultaneous metabolic utilization of the ketoacid into gluconeogenesis pathway for the synthesis of glucose. Archana *et al.* (2007) support the present finding when she observed a decrease in tissue total protein in fresh water teleost, *Channa striatus* (Bloch) when subjected to acute and chronic concentrations of fertilizer industry effluent. Another reason for the decrease in protein level could be the nephrosis or low rate of protein synthesis. This theory has been reported by Lynch *et al.* (1969). The decrease in protein content was accompanied by simultaneous decrease in RNA which clearly suggests the inhibitory effect of effluent at transcription/translation levels. Observations made by Kaur and Kaur (2005) support the present statement. Significant decrease in total protein content indicates that, stress due to ethanol induces proteolysis. Palanichamy *et al.* (1986); Saravanan and Harikrishnan (1997, 1998) have also reported that depletion in protein level was due to the diversification of energy to meet the impending energy demands when the animal was under stress. Protein decrease may be due to stress in fish as protein is likely to undergo hydrolysis and oxidation through TCA cycle to meet the increased demand for energy caused by the stress (Somnath, 1991).

A significant increase ($P<0.001$) (Fig 7.3.15) in the levels of amino acids were observed when *O. mossambicus* was exposed to varying concentrations of ethanol for 7 and 21 days. Increase in free amino acid levels may be the result of breakdown of protein for energy and impaired incorporation of amino acids required in protein synthesis (Singh *et al.*, 1996). Natarajan (1985) suggested that stress conditions induce elevation in the transamination pathway. The present findings were supported by Rao (1984) who found that the decrease in protein moiety was due to the damage brought to the hepatic tissues which finally results in an intensive proteolysis resulting in increased amount of free amino acids which ultimately find their way into TCA cycle as keto acids. The decrease in protein level along with an increase in the levels of free amino acids brings about decrease in the levels of RNA which might indicate an increased catabolism of protein and decreased synthesis. The same findings have been noted by Satyaparameshwar *et al.* (2006) who also observed decrease in total protein levels as well as an increase in free amino acids in

the tissues of fresh water mussels, *Lamellidens marginalis* when being treated with chromium for 72 hours. The decrease in total protein content in the tissues of *O. mossambicus* followed by a concomitant increase in the free amino acid levels in the organs of fresh water teleost, *O. mossambicus* indicate the activation of compensatory mechanisms in the fish to counter the sub lethal toxic stress. Among the organs studied, the liver exhibited greater degree of changes as it is the centre for operation of various metabolic functions. The findings made by Davalli *et al.* (1989) support the present study. An increase in free amino acid content may be the result of tissue damage which too indicates the decreased utilization of amino acids (Seshagiri *et al.*, 1987).

Acid phosphatase is a lysosomal enzyme, which hydrolyses the phosphorous esters in acidic medium and thus, it is logical that the enzyme is hydrolytic in its function and acts as one of the several acid hydrolases in the autolysis process of the cell after its death. Serum enzymes having close relationship to a particular organ or tissue possess high degree of specificity. This enzyme being intracellular is present in small amounts in the blood of healthy animals. But, when there is an increase of cell breakdown due to damage of tissues caused either by disease or pollutants, they escape in greater quantities into the blood with consequent increase in their activities in serum Rajeev *et al.*, 2007). Heart, liver, gills, muscles and kidney are rich in ACP enzyme and their measurement in plasma or serum could often be related to cell damage to specific organ. A significant increase (Fig 7.3.16 and Fig 7.3.17) ($P<0.001$) in acid phosphatase enzyme was observed both in the serum and tissues of *O. mossambicus* when subjected to different sub lethal concentrations of ethanol for 7 and 21 days. This result is in agreement with that of Sastry and Subhadra (1985) who found a significant increase in ACP in kidney of catfish, *Heteropneustes fossilis* on intoxication with cadmium. On the other hand, considerable increase in the levels of acid phosphatase in serum may be due to the leakage of this enzyme from the toxin affected tissues. The findings made by Rajeev *et al.* (2007) supported the above finding.

The present study indicated that sub lethal concentrations of ethanol had deleterious effect on the basic functions of serum, gills, muscle, liver, heart and kidney tissues of *O. mossambicus*. Therefore serum urea, uric acid and creatinine could be used as prognostic indicators of renal dysfunction. Changes in the biochemical parameters thus confirms the stressful condition of fish on exposure to different concentrations of ethanol thus revealing the toxicant nature of the pollutant.

7.4 EFFECT OF ETHANOL ON LIPID METABOLISM

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7.4A Introduction

Due to the enormous growth of technology and industry, pollutants have dramatically amplified in natural environments. In urban and municipal areas seas, rivers and lakes have become a big sink for many man-disposed pollutants (Khadiga *et al.*, 2002). Polluted water habitats exert extensive stress impacts upon aquatic animals. Volatile organic solvents have the potential to change the aquatic medium, affecting the tolerance limit of aquatic fauna and flora, as well as creating danger to the ecosystem.

Metabolism in fish is largely oxidative in nature (Demoute, 1989). The main lipid storage site in fish vary between species, and is located either in the subcutaneous are in the liver, in the muscle myosepta, or in the mesenteric membranes. Lipid is an important fuel reserve of the fish during stress situation, it is mobilized to meet the energy needs. They are essential components of all cells, making up about half the mass of biological membranes and are involved in numerous biological processes. Any change in the composition of lipid reflects

tissue metabolism (Asha and Poonam, 1999). Fishes are sensitive to the contaminations of water, and pollutants significantly damage certain physiological and biochemical processes by entering the organs of these animals (Nemcsok *et al.*, 1987). According to Neff (1985) changes brought about in certain biochemical parameters in fish blood indicate acute or chronic pollutant induced damage.

The cholesterol biosynthetic pathway involves many steps in which the rate limiting enzyme is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. It is the primary regulatory enzyme involved in cholesterol biosynthesis (Hamelin and Turgeon, 1998). Liver is the major organ responsible for cholesterol biosynthesis. HMG-CoA reductase is anchored in the membrane of the endoplasmic reticulum and was long regarded as having seven transmembrane domains, with the active site located in a long carboxyl terminal domain in the cytosol. More recent evidence shows that it contains eight transmembrane domains (Roitelman *et al.*, 1992).

Alterations cited in the circulating levels of total lipids and cholesterol in fish generally reflect the state of the animal as well as the integrity of the vital organs. Triglycerides and cholesterol seems to be the major degradation products and are therefore referred to as indicator of lipid metabolism (Kaplan *et al.*, 1988). Adequate energy (lipid) reserves are required by organisms to mediate the effects of stress (Lee *et al.*, 1983) which in turn serves as energy buffers during the periods of harsh environmental conditions and food shortages (Adams and Mclean, 1985). These considerations indicate that a lipid compartment is important and appears necessary in any individual model that is utilized to represent the biological-chemical interaction (Hallam *et al.*, 1988). Marked alterations in lipid metabolism have been reported in hepatobiliary disorders as well as in chronic ethanol feeding (Hirayama *et al.*, 1979; Weidman *et al.*, 1982). Liver is the most susceptible organ to the toxic effects of alcohol (Lieber and Decarli, 1991). Changes occurring in the biochemical characteristics of fishes provide a sensitive measure to know the health of fish fauna (Nair *et al.*, 1984). The excess intake of alcohol for a long time causes fatty liver and results in the accumulation of fat in the brain, heart and kidneys. Almost all research related to alcohol metabolism has been carried out with pure ethyl alcohol (Indira *et*

al., 2001). Lipids present in the blood are absorbed by liver cells to provide energy for cellular functions. Liver is responsible for providing the proper concentrations of lipids in the blood. Excess lipids in the blood are eventually deposited in adipose tissue. Ethanol is a powerful stimulant of hyperlipidemia in both animals and humans (Avogaro and Cazzolatu, 1975). It also causes a change in the metabolism of lipoproteins (Hirayama *et al.*, 1979). Marked alterations in lipid metabolism have been reported in chronic alcohol feeding (Weidman *et al.*, 1982). The lipid abnormalities seen after alcohol consumption include alterations in the level of cholesterol, fatty acid esters, cholesterol esters and particularly the fatty acyl composition of membrane phospholipids. Lipids are included in cellular structures of all organisms along with proteins, nucleic acids, and carbohydrates the alterations of which get reflected in the membrane architecture (Silkina *et al.*, 2007). It is shown in several studies that the parameters of lipid metabolism including fractional composition of tissue lipids, serves as a reliable diagnostic tool, reflecting the consequences of the influence of different negative factors upon fish health (Lapin and Shatunovskii, 1981; Sidorov, 1983).

7.4B Materials and Methods

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

7.4B.1 Preparation of serum samples for experimental studies

The method remains the same as described in chapter 5, section 5.1B 1

7.4B.2 Methods used for biochemical analysis

The following are the parameters studied under lipid metabolism

a. Estimation of β-hydroxy-β-methylglutaryl-coA reductase (HMG CoA Reductase) activity (E.C 1.1.1.88)

Principle

The activity of HMG-CoA reductase was determined by the method of Rao and Ramakrishnan (1975).

$$HMG\text{-}CoA + 2NADPH + H^{+} \xrightarrow{\text{HMG-CoA reductase}} Mevalonate + CoA + 2NADP^{+}$$

The ratio between HMG-CoA and mevalonate was taken as an index of activity of the enzyme HMG CoA Reductase which catalyses the conversion of HMG-CoA to mevalonate. Lower the ratio, higher the activity of enzyme.

Reagents

1. Saline arsenate: 1 g of sodium arsenate was dissolved in 1L of physiological saline.
2. Dilute perchloric acid ($HClO_4$): 50 ml of Conc. $HClO_4$ was made up to 1 litre with Dist. H_2O.
3. Hydroxylamine hydrochloride reagent: 138.98 g of hydroxyl amine hydrochloride was weighed and made to 1 litre.
4. Hydroxylamine hydrochloride reagent for mevalonate: Equal volumes of hydroxylamine hydrochloride and water were mixed freshly before use.
5. Hydroxylamine hydrochloride reagent for HMG-CoA: Equal volumes of hydroxylamine hydrochloride and sodium hydroxide solution (1.8g/L) were mixed before use.
6. Ferric chloride reagent ($FeCl_3$): 5.2 g of TCA and 10 g of $FeCl_3$ were dissolved in 50 ml of 0.65N hydrochloric acid (HCl) and were made upto 100 ml with water

Procedure

The tissues were removed as quickly as possible and a 10% homogenate of gill and muscle, followed by 5% homogenate of liver and 1% homogenate of heart and kidney tissues were prepared using saline arsenate. The homogenate was deproteinized using an equal volume of dilute perchloric acid. It was then allowed to stand for five minutes. The contents were then centrifuged at 1000g for 15 minutes. The supernatants were used for the assay. To 1ml of the filtrate 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG CoA) was added and mixed. The tubes were allowed to stand for 5 minutes. After keeping for 5 minutes, 1.5 ml of ferric chloride reagent was added. After

suitable shaking and keeping for 10 minutes, optical density at 540 nm against a similarly treated saline arsenate as blank was measured. The ratio of HMG CoA to mevalonate was taken as an index of enzyme activity which catalyses the conversion of HMG CoA to mevalonate in the presence of HMG Co A Reductase. Lower ratio indicates higher enzymic activity.

b. Extraction of lipids

Reagents

1. 0.1 N KCl
2. Folch reagent – This reagent was made by using 0.1 N KCl: Methanol: chloroform in the ratio 10:10:1v/v.

Lipids were extracted from liver and muscle tissues by the method of Folch *et al.* (1957) using chloroform-methanol mixture (2:1 v/v). The tissues were washed in saline (0.9% NaCl) solution and dried using filter paper. Pooled liver and muscle samples taken from *O. mossambicus* after suitable exposure periods were taken for lipid estimation. A known weight of tissue samples were homogenized in 7.0 ml of chloroform-methanol mixture. The homogenized contents were then filtered through a Whatman No.1 filter paper into a conical flask which was previously weighed. The residue on the filter paper was scraped off and was again homogenized with 14 ml of chloroform-methanol mixture. This was again filtered into the side arm flask and the residue was successively homogenized in chloroform-methanol (2:1 v/v) and the extract was filtered each time. The pooled filtrate in the flask was adjusted to a final volume ratio using chloroform-methanol (2:1 v/v) and evaporated to dryness to a constant weight. The dried residue of lipid was dissolved in 5 ml of chloroform-methanol mixture (2:1 v/v) and was transferred into a centrifuge tube, 2 ml of 0.1N potassium chloride was added, shaken well and centrifuged. The upper aqueous layer containing gangliosides were discarded. The lower chloroform layer was mixed with 1.0 ml of chloroform-methanol-potassium chloride mixture (1:10:10 v/v) and was then centrifuged. This washing was carried out for three times. The upper layer was discarded. The lower layer was then made upto 5.0 ml and was used for the analysis of total lipids, total cholesterol, triglycerides, free fatty acids and phospholipids.

c. Estimation of total lipid

Principle

Total lipids present in serum and tissue homogenates were estimated by sulpho-vanillin reaction of Chabrol and Charronat as given by Choudhary (1989).

Lipids on heating with concentrated sulphuric acid (H_2SO_4) gets oxidized to ketones which forms pink color with phosphoric acid and vanillin.

Reagents

1. Vanillin – 0.65%: 650 mg vanillin was dissolved in 100 ml of 1% benzoic acid solution.
2. Conc. Sulphuric acid (H_2SO_4): A/R grade.
3. Conc. Phosphoric acid (H_3PO_4): A/R grade.
4. Standard lipid solution: 1 g of pure groundnut oil was dissolved in chloroform-methanol and was made to 100 ml with chloroform.

Procedure

1ml of chloroform-methanol (1:1) extract in the case of tissues followed by 0.01 ml of serum sample was taken in a test tube. The content present in the tube was evaporated to dryness using a water bath maintained at 70^0C. All the tubes were then cooled for 5 minutes. To this 1 ml of concentrated sulphuric acid was added. Simultaneously into another test tube 1 ml of vanillin followed by 4 ml of phosphoric acid was added. The tube was then mixed well. Then to this tube 0.1 ml of sulphated extract or serum was added. It was then shaken vigorously and were incubated at 37^0C for 15 minutes. After suitable period of incubation the tubes were removed and were cooled to room temperature. The absorbance was then read against blank at 540 nm. For standard 0.1 ml of standard lipid solution and for blank 0.1 ml distilled water was used in place of test sample in the procedure. Values were expressed as mg/100 ml in the case of serum and mg/g in the case of tissues.

d. Estimation of triacylglycerol (TAG)

Principle

TAG was estimated by the method of Hantzsh reaction as given by Foster and Dunn, 1973; Fletcher, 1968). Phospholipids were removed using alumina-isopropanol mixture. In this, alumina serves as an adsorbent for phospholipids; and TAG were extracted into isopropanol after saponification with potassium hydroxide, to yield glycerol and soap. The glycerol liberated after saponification was treated with metaperiodate, which released formaldehyde, formic acid and iodide upon oxidation. The formaldehyde released reacts with acetyl acetone and ammonia forming a yellow coloured compound, the intensity of which was measured at 405 nm.

Reagents

1. Isopropanol.
2. Alumina-activity grade I: Washed with water until all fines were removed and dried in an oven overnight.
3. Saponifying reagent: Dissolved 50 g potassium hydroxide in 600 ml water and added 400 ml of isopropanol to it.
4. Sodium meta periodate reagent: Dissolved 77 g of anhydrous ammonium acetate in about 700 ml water. To this 60 ml of glacial acetic acid was added followed by 650 mg of sodium meta periodate. The mixture was then made up to 1 L with water.
5. Acetyl acetone reagent: Added 7.5 ml of acetyl acetone to 200 ml of isopropanol. The mixture was mixed and to this 800 ml water was added.
6. Stock triolein solution: Accurately weighed 8.85 g of triolein. It was then dissolved in 1 litre of isopropanol.
7. Working triolein solution: 1.0 ml of stock standard was diluted to 100ml to prepare a working standard of concentration 100µg of triolein/ml.

Procedure

0.1ml of the sample lipid extract and 0.01 ml of serum sample was taken. Added 0.4 ml of distilled water followed by 4 ml isopropanol to each of the tubes. The tubes were mixed well and 400 mg washed alumina was added to it. It was placed in a rotator and was centrifuged for 15 minutes. After centrifuging the supernatant was transferred to another tube. To 2 ml supernatant, 0.8 ml of saponifying reagent was added. The test tubes were incubated at 60 – 70^0C. All the tubes were cooled. To this 1 ml of metaperiodate solution was added. It was then mixed well, and 0.5 ml of acetyl acetone reagent was added. Mixed and stoppered the test tube. The tubes were then incubated at 50^0C for 30 minutes. For blank, 0.1 ml of water was treated in the same manner similar to the test. The absorbance was then read against blank at 405 nm. Values were expressed as mg/100 ml in the case of serum and mg/g wet weight in the case of tissues.

e. Estimation of total cholesterol

Principle

Total cholesterol was estimated by the method of Zak *et al.* (1953) using ferric chloride-sulphuric acid method. Sample is treated with ferric chloride-acetic acid reagent to precipitate the protein. The protein free filtrate containing cholesterol ferric chloride was treated with Conc. H_2SO_4. The reaction involves the 3-hydroxy-5-ene part of the cholesterol molecule, which was first dehydrated to form cholesta-3,5-diene and then oxidized by sulphuric acid to link two molecules together as bis-cholesta-3,5diene. This material can be sulphonated by sulphuric acid to produce the highly red coloured mono-and di-sulphonic acids in the presence of ferric ion as catalyst (Solkowski's reaction). The colour developed was read at 560 nm using suitable standard and a reagent as blank.

Reagents

1. Glacial acetic acid – A/R grade.
2. Ferric chloride-acetic acid ($FeCl_3$-CH_3COOH) reagent: 0.5% solution of ferric chloride ($FeCl_3$. $6H_2O$) was prepared in purified acetic acid.

3. Conc. H_2SO_4 - A/R grade.
4. Stock standard solution: Dissolved 100 mg of cholesterol in 100 ml of glacial acetic acid.
5. Working standard: 5 ml of stock standard was diluted to 100 ml with ferric chloride acetic acid ($FeCl_3$-CH_3COOH) reagent.

Procedure

To 0.1 ml of the tissue lipid extract sample and 0.01 ml of serum, 9.9 ml of $FeCl_3$-CH_3COOH reagent was added. The tubes were mixed well. They were allowed to stand for 15 minutes for the proteins to flocculate. The mixture was centrifuged and 5 ml of supernatant was transferred to another tube. For standard, 2 ml of working standard was taken. It was then made to 5 ml with $FeCl_3$-CH_3COOH reagent. For blank 5 ml $FeCl_3$-CH_3COOH reagent was taken. Then to all the tubes, 3 ml of Conc. H_2SO4 was added slowly. The contents present in the tube was mixed well. The standard and test were read against blank at 560 nm using a colorimeter. Values were expressed as mg/100 ml in the case of serum and mg/g wet weight in the case of tissues.

f. Estimation of HDL cholesterol

Principle

HDL cholesterol was estimated by phosphotungstate/magnesium method adopted by Burstein, *et al.* (1970). LDL, VLDL, chylomicrons are precipitated by polyanions in the presence of metal ions to leave HDL in solution. 3-hydroxy-5-ene part of the HDL cholesterol molecule is dehydrated and then oxidized by concentrated sulphuric acid. This material can be sulphonated by sulphuric acid to produce the highly coloured mono – and - di-sulphonic acids.

Reagents

1. Phosphotungstate reagent: Dissolved 22.5 g of phosphotungstic acid (AR grade) in 200 ml water. To this added 80 ml of 1 mol/L NaOH and was made up to 500 ml with water.

2. Magnesium chloride solution – 2 mol/L: Dissolved 101.7 g $MgCl_2.6H_2O$ in water and was made up to 250 ml.
3. Glacial acetic acid – A/R grade.
4. Ferric chloride acetic acid reagent.
5. Conc. H_2SO_4– A/R grade.
6. Stock standard solution: 100 mg cholesterol was dissolved in 100 ml of purified acetic acid.
7. Working standard: 1 ml of the stock solution was made up to 25 ml with $FeCl_3$-CH_3COOH reagent.

Procedure

Added 0.1ml of phosphotungstate reagent and 0.05 ml of $MgCl_2$ to 0.01ml of the serum sample in a centrifuge tube. The tubes were mixed well. It was then centrifuged at 2500 rpm for 30 minutes. Supernatant was taken. To 0.1 ml of the supernatant, 10 ml of $FeCl_3.CH_3COOH$ reagent was added. The contents of the tube were mixed well. It was then kept undisturbed for 10 -15 minutes for the proteins to flocculate. The tubes were again centrifuged. 5 ml of clear supernatant was taken which is labelled as 'Test'. 5 ml of $FeCl_3$-CH_3COOH reagent was taken as blank. For standard, 1 to 5 ml of cholesterol working standard was taken. It was then made up to 5 ml using $FeCl_3$-CH_3COOH reagent. Added 5 ml of concentrated sulphuric acid to all of the tubes. The tubes were then allowed to stand for 20-30 minutes. The colour developed was read colorimetrically at 560 nm. Values were expressed as mg/dl in the case of serum.

g. Estimation of LDL+ VLDL cholesterol

LDL + VLDL cholesterol was determined by subtracting HDL cholesterol from total cholesterol.

LDL + VLDL cholesterol = total cholesterol-HDL cholesterol.

h. Estimation of free fatty acids

Principle

Non – esterified free fatty acids were estimated by the method of Horn and Mehanan (1981). The free fatty acids extracted from lipids by chloroform-heptane-methanol (CHM) mixture forms a complex with cupric ions when mixed with copper reagent. The coloured complex formed with copper is soluble in chloroform and diethyl dithiocarbamate which is used as colour developer. The colour developed was read at 660 nm.

Reagents

1. Chloroform-heptane-methanol (CHM mixture): The mixture was prepared in the ratio of 200:150 :7 (v/v)
2. Activated silicic acid
3. Copper nitrate-triethanolamine solution: 9 volumes of aqueous 1 M triethanolamine, 1 volume of 1 N acetic acid and 20 volumes of 6.45% $Cu(NO_3)_2$ $3H_2O$ were mixed with 33 g of sodium chloride. The pH was adjusted to 8.1.
4. 0.1 % diethyl dithiocarbamate in n-butanol
5. Stock standard: A solution containing 200 mg/100ml of palmitic acid was prepared in CHM mixture.
6. Working standard: The stock solution was diluted 1 in 10 times for use (200 µg/ml).

Procedure

To 0.2 ml of lipid extract and 0.01 ml of the serum sample, 6.0 ml of CHM mixture and 200 mg of activated silicic acid were added. The tubes were mixed well and centrifuged. Supernatant was taken. It was then transferred to another tube. Standards were also made up to 6.0ml with CHM mixture. Blank contained 6.0ml of CHM mixture alone. To all these tubes, 2.0 ml of copper nitrate - TAE solution was added. It was then mixed on a mechanical shaker for 20 minutes. They were then centrifuged to give two separate phases. To 2.0 ml of the upper phase, 1.0 ml of the

colour reagent was added, and shaken well. The colour developed was read at 430 nm against a reagent blank. Free fatty acids were expressed as mg/100 ml in serum and mg/g in tissues.

i. Estimation of Phospholipids

Principle

Phospholipids present in the serum and tissue extract were estimated according to the method of Rouser *et al.*, 1970). The organic phospholipid phosphorus is converted to inorganic phosphorus which reacts with ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with ANSA forms a stable blue colour that has an absorption maximum at 660nm.

Reagents

1. 70% Perchloric acid
2. 3% Ammonium molybdate
3. 3% Ascorbic acid
4. Standard: 35.1 mg of KH_2PO_4 was dissolved in 100ml of water. This contains 80 µg of phosphorus/ml.

Procedure

To 0.1 ml of lipid extract and 0.01 ml of serum, 1.0 ml of perchloric acid was added and digested on a sand bath until it become colourless. The volume was made upto 5.0 ml with water. Blank containing 0.8 ml of perchloric acid and 4.2 ml of water was taken. Standards in the range 5-20 µg were also taken and 0.8 ml of perchloric acid was added and the contents were made upto 5.0 ml with water. To all the tubes, 0.5 ml of ammonium molybdate was added followed by 0.5 ml of ascorbic acid solution. The contents were mixed well. The tubes were heated in a boiling water bath for 6 min and the colour developed was read immediately at 700 nm. Phospholipid concentration was expressed as mg/100 ml in serum and mg/g in tissues.

j. Determination of serum lipase (Triacylglycerol acylhydrolase, E.C 3.1.1.3)

Lipase activity in the serum sample was carried out by the method of Cherry and Crandall (1932). Lipase hydrolyses the fats into fatty acid and glycerol. The fatty acids liberated are determined by titration with standard NaOH. Fat emulsion such as olive oil was used as substrate. In the titrimetric method the fatty acids liberated in the reaction were titrated with standard 0.05 N NaOH using phenolphthalein as an indicator. The amount of NaOH used to neutralize the fatty acids liberated seems to be equivalent to the units of lipase present in the sample.

Reagents

1. Olive oil (50%) emulsion: Homogenized a mixture of equal parts of olive oil and a 5% solution of gum acacia containing 0.2% of sodium benzoate as preservative.
2. Phosphate buffer, pH 7: Mixed 100 ml of M/3 disodium hydrogen phosphate (47.3 grams of Na_2HPO_4 per litre) and 30 ml of M/3 potassium dihydrogen phosphate (45.3 grams KH_2PO_4) per litre.
3. 95% ethanol
4. 0.05 N NaOH
5. Phenolphthalein: 1% solution was dissolved in ethanol.

Procedure

3 ml of distilled water was pipetted into two tubes. Added 1 ml of serum to each of the tubes. Placed one in a water bath at 100^0 C for 5 minutes in order to inactivate the lipase. The tubes were then cooled. To both the tubes added 0.5 ml of the buffer solution followed by the addition of 2 ml of the 50% olive oil emulsion. The tubes were shaken well. It was then incubated at 37^0C for twenty four hours. Then, after adding 3 ml of 95 % ethanol and 2 drops of phenolphthalein, the tubes were titrated against 0.05 N NaOH to obtain a similar pink colour.

7.4C Results

Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l concentrations of ethanol in *O. mossambicus* exposed for 7 and 21 days exhibited marked alterations in the serum and tissue lipid profiles depicted in table 7.4.1 to 7.4.24 and in figures 7.4.3 to 7.4.22. Results obtained were then statistically analyzed by ANOVA (Analysis of Variance) followed by Tukey's test and Dunnett's method.

Table 7.4.1 Effect of exposure to different concentrations of ethanol for 7 days on the levels of HMG CoA Reductase activity in different tissues of *O. mossambicus*.

Parameter Investigated	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
HMG CoA Reductase	Gills	0.623± 0.0163	0.585± 0.0105	0.528± 0.0194	0.457± 0.0175
	Muscle	0.678± 0.0214	0.622± 0.0147	0.562± 0.0075	0.495± 0.0105
	Liver	0.893± 0.0137	0.850± 0.0089	0.813± 0.0082	0.777± 0.0151
	Heart	0.785± 0.0105	0.677± 0.0137	0.618± 0.0172	0.520± 0.0141
	Kidney	0.632± 0.0232	0.578± 0.0117	0.523± 0.0197	0.473± 0.0197

Values are expressed as the ratio of HMG CoA/Mevalonate

Average of six values± SD of six observations

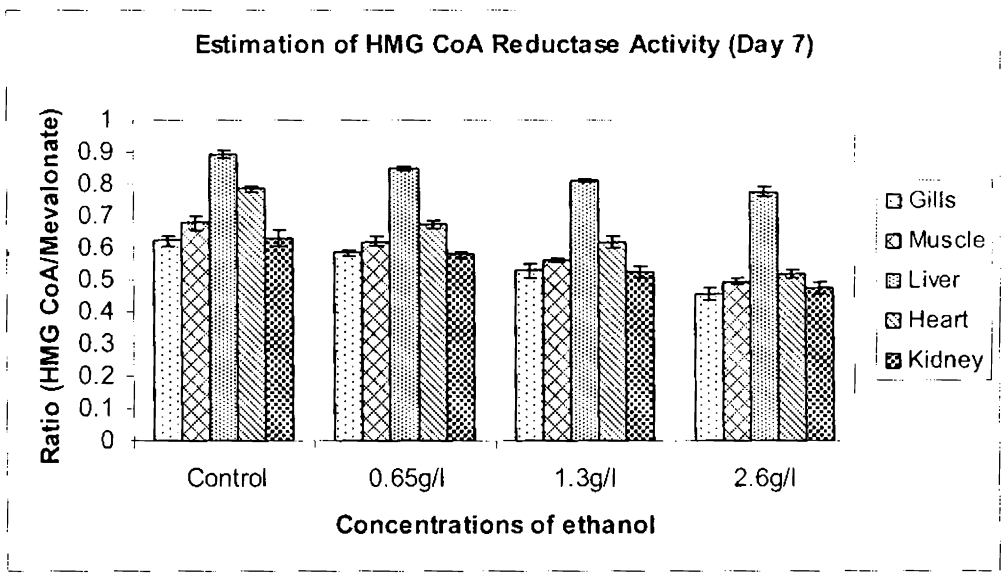
Table 7.4.2 Effect of exposure to different concentrations of ethanol for 21 days on the levels of HMG CoA Reductase activity in different tissues of *O. mossambicus*.

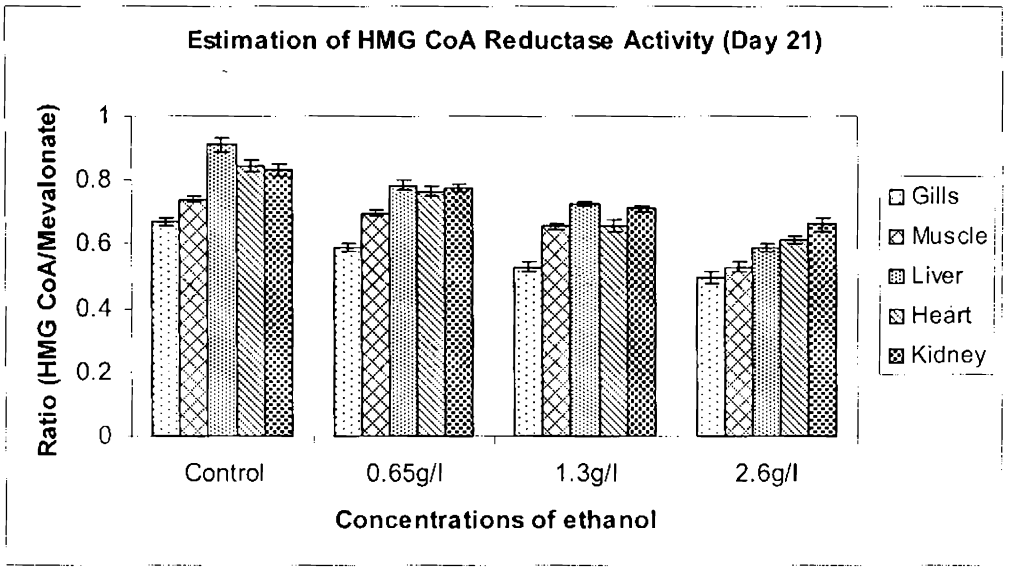
Parameter Investigated	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
HMG CoA Reductase	Gills	0.670± 0.0141	0.592± 0.0117	0.530± 0.0141	0.495± 0.0187
	Muscle	0.740± 0.0089	0.698± 0.0075	0.657± 0.0082	0.530± 0.0155
	Liver	0.910± 0.0237	0.785± 0.0152	0.727± 0.0052	0.590± 0.0141
	Heart	0.845± 0.0207	0.767± 0.0163	0.658± 0.0172	0.615± 0.0152
	Kidney	0.833± 0.0175	0.778± 0.0117	0.712± 0.0117	0.662± 0.0214

Values are expressed as the ratio of HMG CoA/Mevalonate

Average of six values± SD of six observations

Figure 7.4.3 Levels of HMG CoA Reductase activity in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant increase ($P<0.001$) in HMG CoA Reductase activity was noted in *O. mossambicus* exposed to the three sub lethal concentrations of ethanol as compared to control group (Figure 7.4.3). Investigations using ANOVA substantiates the above statement and the results are shown below (Table 7.4.3a).

Table 7.4.3a ANOVA table for tissue HMG CoA reductase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.183	1	0.183	66.094	0.000
Between Concentrations	1.305	3	0.435	157.500	0.000
Between Tissues	1.434	4	0.358	129.739	0.000
Error	0.638	231	0.003		
Total	3.560	239			

df- degrees of freedom

Statistical analysis done by using Three Factor ANOVA revealed that HMG CoA Reductase activity levels varied significantly between days ($P<0.001$). It was noted that between concentrations there was significant difference ($P<0.001$). Also when tissues were taken into consideration significant difference ($P<0.001$) was obtained.

Table 7.4.4 Multiple Comparison Test (Concentrations)

After employing Three Factor ANOVA, subsequent pair wise comparisons between concentrations done by multiple comparison tests using Dunnett's is shown below

	Groups	Sig.
Dunnett	Control Vs 0.65g/l	0.000[a]
	Control Vs 1.3g/l	0.000[a]
	Control Vs 2.6g/l	0.000[a]

The values are significant at a = P < 0.001.

Table 7. 4.5 Multiple Comparison Test (Tissues)

Subsequent comparisons between tissues by multiple comparison tests using Tukey is shown below

	Tissues	Sig.
Tukey	Gills Vs Muscle	0.000[a]
	Gills Vs Liver	0.000[a]
	Gills Vs Heart	0.000[a]
	Gills Vs Kidney	0.000[a]
	Muscle Vs Liver	0.000[a]
	Muscle Vs Heart	0.000[a]
	Muscle Vs Kidney	0.107[d]
	Liver Vs Heart	0.000[a]
	Liver Vs Kidney	0.000[a]
	Heart Vs Kidney	0.007[b]

The values are significant at a = P < 0.001, b = P < 0.01 and not significant at d.

Pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method. In the case of HMG Co A Reductase values

with respect to control, significant difference (P<0.001) was obtained for all the three sub lethal concentrations of ethanol used, such as 0.65g/l, 1.3g/l and 2.6g/l. For comparing pair wise comparison for tissues Tukey was used. Tissues such as muscle, liver, heart and kidney when being compared with gills exhibited significant difference (P<0.001). Similarly liver and heart tissues when compared with muscle also exhibited significant difference (P<0.001). Kidney on comparison with muscle tissue exhibited no significant difference (NS). Significant difference (P<0.001 was obtained when heart and kidney tissues were compared with liver. It was seen that Kidney when compared with heart showed significant difference (P<0.01).

Table 7.4.6 Effect of exposure to different concentrations of ethanol for 7 days on lipid profile parameters of *O. mossambicus*.

Parameters Investigated	Sample Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Total Lipids	Serum	6.254± 0.0076	6.448± 0.0042	6.524± 0.0039	6.683± 0.0019
	Liver	6.349± 0.0048	6.857± 0.0017	6.303± 0.0033	6.195± 0.0024
	Muscle	3.937± 0.0225	4.425± 0.0176	4.192± 0.0172	3.987± 0.0327
Phospholipids	Serum	1.163± 0.1019	2.263± 0.0516	2.267± 0.1091	3.377± 0.1285
	Liver	1.278± 0.0263	2.440± 0.0420	2.165± 0.1124	3.013± 0.0784
	Muscle	0.273± 0.0121	0.675± 0.0327	0.333± 0.0197	0.235± 0.0207
Fatty Acids	Serum	4.918± 0.0117	4.988± 0.0194	5.037± 0.0082	5.095± 0.0105
	Liver	4.365± 0.0274	4.878± 0.0214	4.220± 0.0167	4.090± 0.0358
	Muscle	8.353± 0.0294	15.64± 0.8133	8.887± 1.0623	6.182± 0.2313
Lipase	Serum	4.893± 0.0082	4.988± 0.0098	5.007± 0.0082	5.052± 0.0098

Average of six values± SD of six observations

Values are expressed as mg/dl in serum and mg/g in tissue

Table 7.4.7 Effect of exposure to different concentrations of ethanol for 7 days on lipid profile parameters of *O. mossambicus*.

Parameters Investigated	Sample Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Triglycerides	Serum	33.17± 0.8319	36.50± 0.4591	38.22± 0.2469	44.33± 1.2111
	Liver	5.196± 0.0109	5.021± 0.0153	4.747± 0.0223	4.616± 0.0407
	Muscle	3.510± 0.0352	4.845± 0.0138	4.373± 0.0307	4.282± 0.0160
HDL Cholesterol	Serum	64.72± 1.3910	69.56± 1.3599	72.47± 0.8043	75.30± 0.6491
Total Cholesterol	Serum	56.07± 0.7640	41.17± 0.7528	34.00± 0.6325	28.83± 0.9832
	Liver	42.74± 0.9050	70.54± 1.9322	55.37± 2.0630	47.17± 1.7952
	Muscle	5.880± 0.3989	13.26± 0.7312	6.690± 0.2419	4.722± 0.2177
LDL + VLDL Cholesterol	Serum	11.58± 1.7882	26.59± 2.8715	34.99± 3.1717	44.51± 2.2014

Average of six values± SD of six observations

Values are expressed as mg/dl in serum and mg/g in tissue

Table 7.4.8 Effect of exposure to different concentrations of ethanol for 21 days on lipid profile parameters on *O. mossambicus.*

Parameters Investigated	Sample Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Total Lipids	Serum	6.346± 0.0031	6.134± 0.0064	5.827± 0.0107	5.641± 0.0091
	Liver	6.399± 0.0044	6.478± 0.0034	6.450± 0.0044	6.433± 0.0038
	Muscle	3.970± 0.0210	4.407± 0.0345	4.108± 0.0264	4.190± 0.0335
Phospholipids	Serum	4.900± 0.1526	5.712± 0.0366	6.510± 0.0456	6.912± 0.0588
	Liver	0.460± 0.0141	0.852± 0.0306	1.258± 0.0445	2.608± 0.0343
	Muscle	0.250± 0.0072	0.282± 0.0075	0.348± 0.0117	0.582± 0.0147
Fatty Acids	Serum	5.112± 0.0098	4.097± 0.0163	4.977± 0.0121	4.995± 0.0055
	Liver	3.388± 0.0818	3.692± 0.1042	4.213± 0.0225	1.288± 0.0264
	Muscle	9.263± 0.2517	7.362± 0.1212	6.792± 0.1859	6.077± 0.0745
Lipase	Serum	5.912± 0.0041	4.928± 0.0098	4.970± 0.0089	5.022± 0.0117

Average of six values ± SD of six observations

Values are expressed as mg/dl in serum and mg/g in tissue

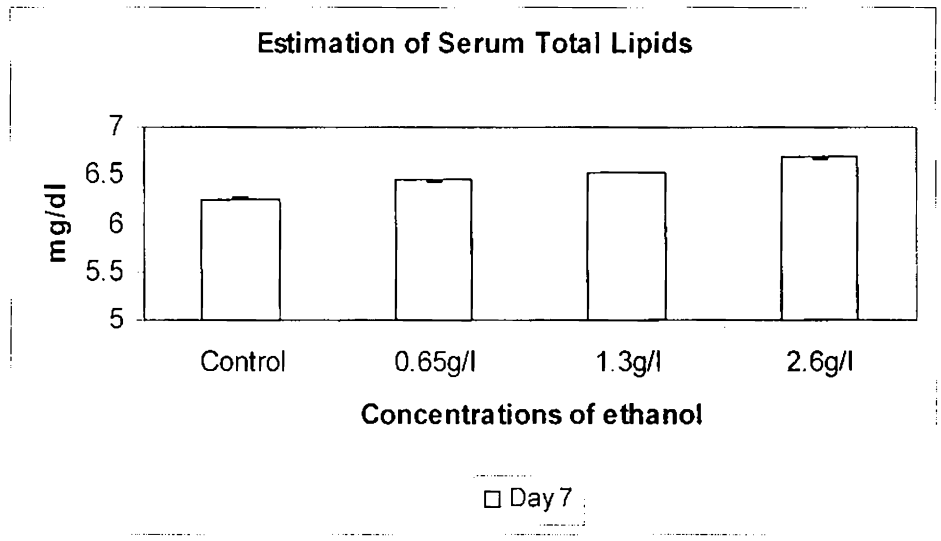
Table 7.4.9 Effect of exposure to different concentrations of ethanol for 21 days on lipid profile parameters on *O. mossambicus*.

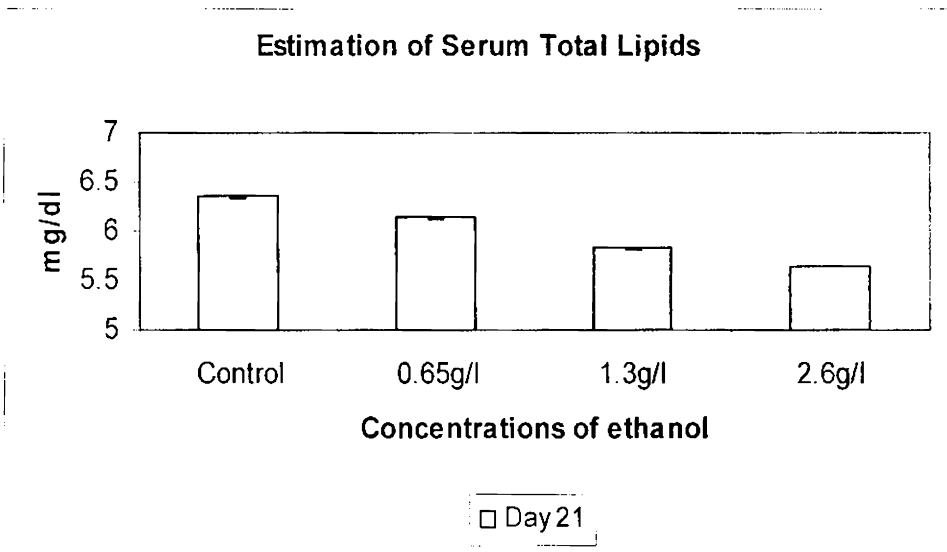
Parameters Investigated	Sample Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Triglycerides	Serum	37.52± 0.5244	34.13± 0.6455	24.92± 0.8010	23.00± 0.8944
	Liver	4.083± 0.0184	4.608± 0.0117	4.672± 0.0117	4.835± 0.0055
	Muscle	3.848± 0.0571	4.040± 0.0179	4.125± 0.0217	4.275± 0.0288
HDL Cholesterol	Serum	79.61± 0.6581	76.58± 0.8604	75.53± 1.5487	65.00± 1.2537
Total Cholesterol	Serum	55.91± 0.7755	46.09± 0.6871	41.83± 0.4083	29.44± 0.9816
	Liver	17.02± 2.5356	45.72± 0.9572	30.40± 0.9334	27.53± 0.7611
	Muscle	4.368± 0.2690	5.093± 0.1071	10.07± 0.6802	13.17± 0.4959
LDL + VLDL Cholesterol	Serum	19.31± 1.8671	28.45± 1.4416	32.36± 1.7054	41.16± 3.1822

Average of six values± SD of six observations

Values are expressed as mg/dl in serum and mg/g in tissue

Figure 7.4.10 Levels of total lipids in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Significant differences ($P<0.001$) were observed in the serum total lipid level of *O. mossambicus* exposed to 7 and 21 days (Figure 7.4.10). Statistical approach employing ANOVA corroborates this and can be inferred from the table below (Table 7.4.10a).

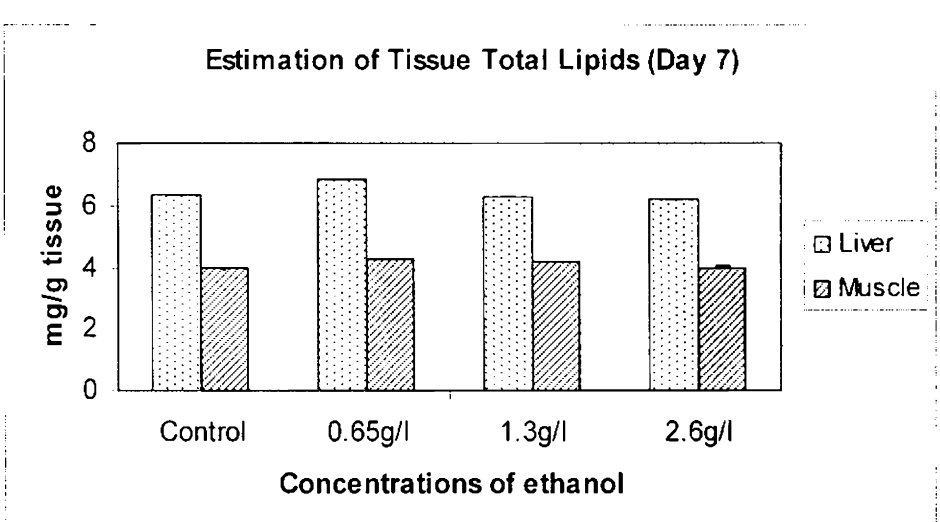
7.4.10a ANOVA table for serum total lipids

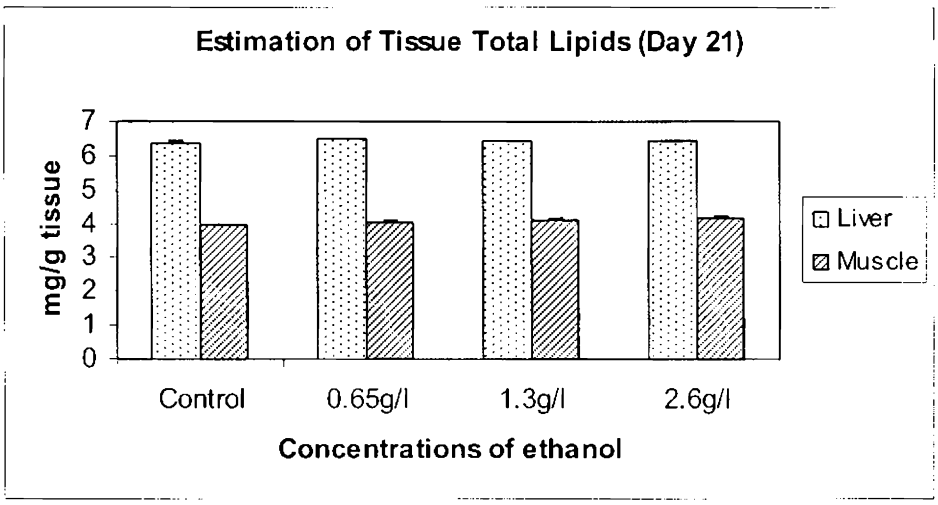
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	2.887	1	2.887	67007.405	0.000
Between Concentrations	0.194	3	0.065	1500.420	0.000
Days of Exposure × concentration	2.151	3	0.717	16643.249	0.000
Error	0.002	40	4.31E-005		
Total	5.234	47			

df-degrees of freedom

Studies done by using Two Factor ANOVA indicated that total lipid levels in the serum varied significantly between days ($P<0.001$). There was significant difference ($P<0.001$) between concentrations also. While comparing both the days as well as concentrations (interaction), significant difference ($P<0.001$) was noted.

Figure 7.4.11 Levels of total lipids in the tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





O. mossambicus exposed to various sub lethal concentrations of ethanol exhibited critical changes in the tissue total lipid levels (Figure 7.4.11). Employing ANOVA supports the above statement (Table 7.4.11a).

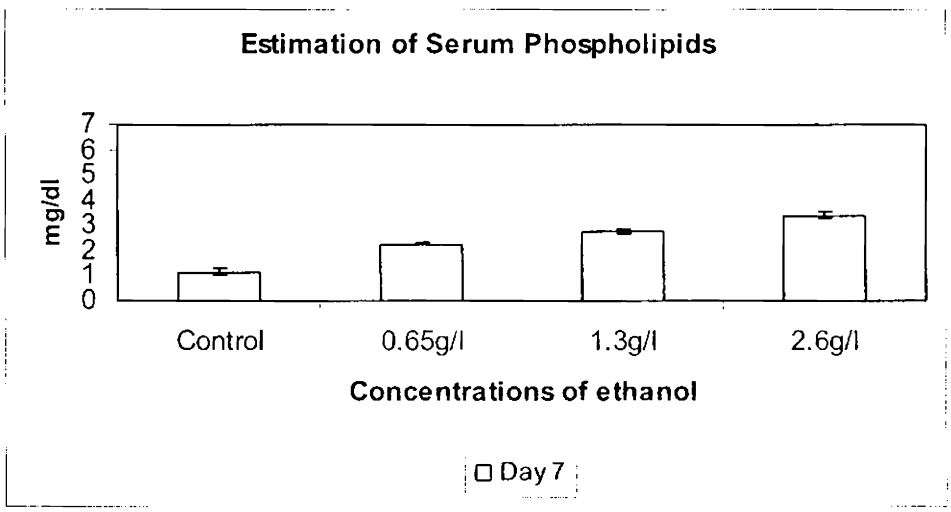
Table 7.4.11a ANOVA Table for tissue Total Lipids

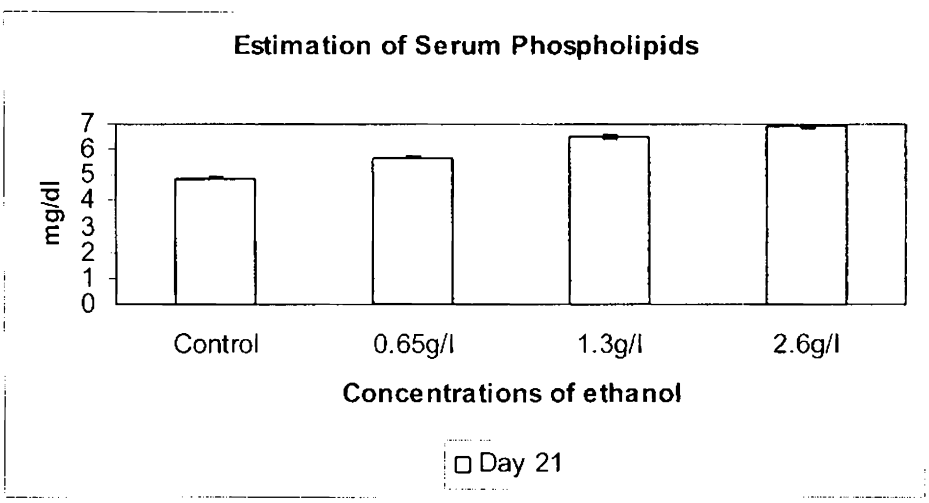
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	4.90E-005	1	4.90E-005	0.003	0.955
Between Concentrations	0.823	3	0.274	18.103	0.000
Between Tissues	132.516	1	132.516	8747.582	0.000
Error	1.363	90	0.015		
Total	134.702	95			

df-degrees of freedom

Statistical analysis carried out by using Three Factor ANOVA table indicated that in the case of tissue total lipids, while considering the exposure between days no significant difference was observed. While taking into consideration the effect between concentrations significant difference ($P<0.001$) was noted. Finally when tissues were taken into consideration, significant difference ($P<0.001$) was observed.

Figure 7.4.12 Levels of phospholipids in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





A significant increase ($P<0.001$) in phospholipid was noted in the serum of *O. mossambicus*, treated with various sub lethal concentrations of ethanol, with respect to control during 7 and 21 days of exposure period (Figure 7.4.12). ANOVA has been carried out to ascertain the statement and the table is shown below (Table 7.4.12a).

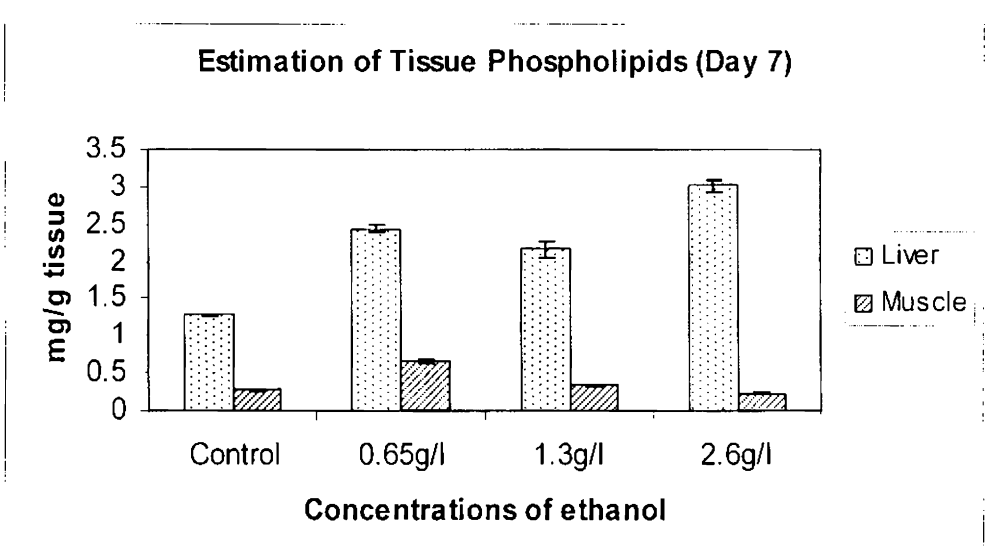
Table 7.4.12a ANOVA table for serum phospholipids

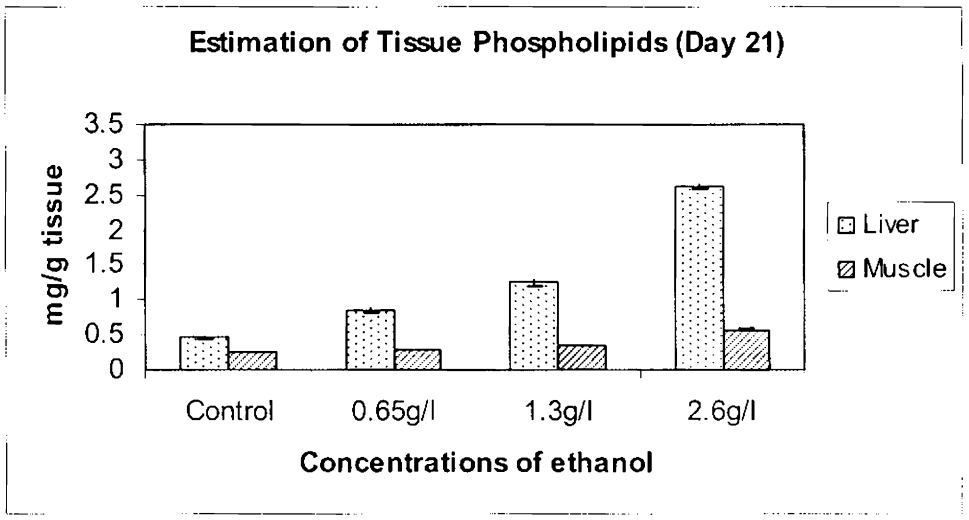
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	156.891	1	156.891	24295.936	0.000
Between Concentrations	29.925	3	9.975	1544.712	0.000
Days of Exposure × Concentration	0.196	3	0.065	10.135	0.000
Error	0.258	40	0.006		
Total	187.270	47			

df-degrees of freedom

By using Two Factor ANOVA it was noted that serum phospholipid levels varied significantly between days ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the days as well as concentrations (Interaction), significant difference ($P<0.001$) was observed.

Figure 7.4.13 Levels of phospholipids in the tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Relevant significant (P<0.001) changes were noted in the tissue phospholipid levels (Figure 7.4.13) in *O. mossambicus* exposed to 7 and 21 days of exposure period. Analysis using ANOVA authenticate this (Table 7.4.13a).

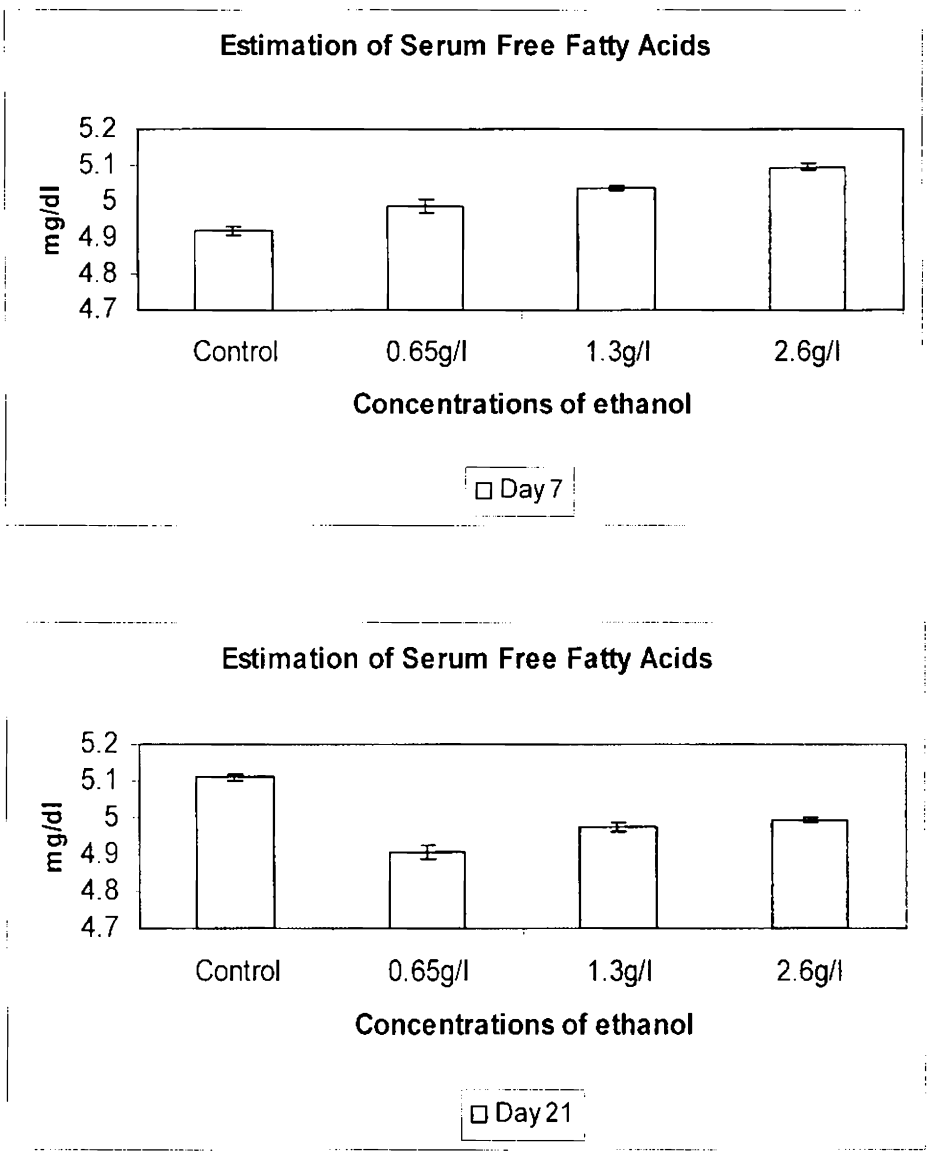
Table 7.4.13a ANOVA table for tissue phospholipids

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	5.341	1	5.341	26.289	0.000
Between Concentrations	13.147	3	4.382	21.572	0.000
Between Tissues	46.180	1	46.180	227.320	0.000
Error	18.284	90	0.203		
Total	82.952	95			

df-degrees of freedom

Three factor ANOVA table indicated that while considering the tissue phospholipid levels, between days of exposure significant difference (P<0.001) was obtained. When taken the effect between concentrations significant difference (P<0.001) was noted. Finally, between tissues also, significant difference (P<0.001) was observed.

Figure 7.4.14 Levels of free fatty acids in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Significant difference ($P<0.01$) in serum free fatty acid level (Figure 7.4.14) was observed in *O. mossambicus* exposed to all the three sub lethal concentrations of ethanol with respect to control group. To validate this ANOVA was carried out and the results are depicted below (Table 7.4.14a).

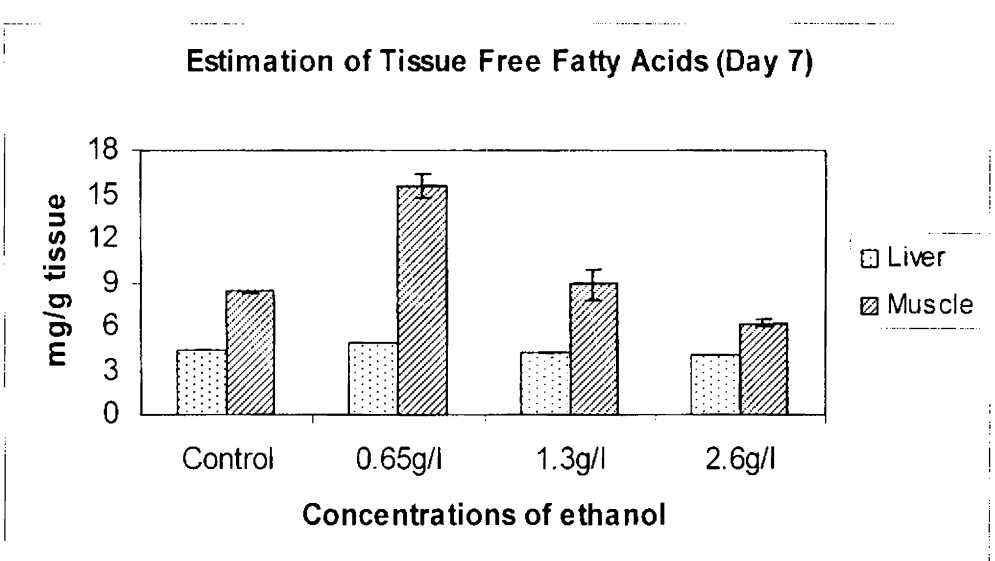
Table 7.4.14a ANOVA table for serum free fatty acids

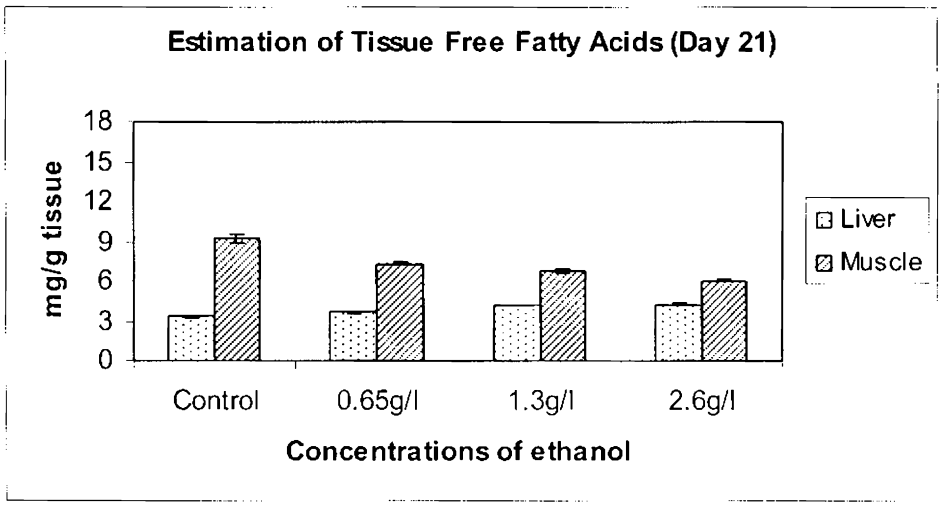
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.002	1	0.002	11.312	0.002
Between Concentrations	0.060	3	0.020	133.319	0.000
Days of Exposure × Concentration	0.173	3	0.058	385.117	0.000
Error	0.006	40	0.000		
Total	0.241	47			

df-degrees of freedom

Two Factor ANOVA table indicated that serum free fatty acid levels varied significantly ($P<0.01$) between days. Between concentrations, a marked significant difference ($P<0.001$) was noted. After considering the interaction effects of both the days as well as concentrations significant difference ($P<0.001$) was seen.

Figure 7.4.15 Levels of free fatty acids in the tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Highly significant (P<0.001) alterations were observed in tissue free fatty acid levels in different sub lethal concentrations of the treatment group when compared to control group of *O. mossambicus* during exposure periods of 7 and 21 days (Figure 7.4.15).Statistical approach using ANOVA substantiates the above mentioned statement (Table 7.4.15a).

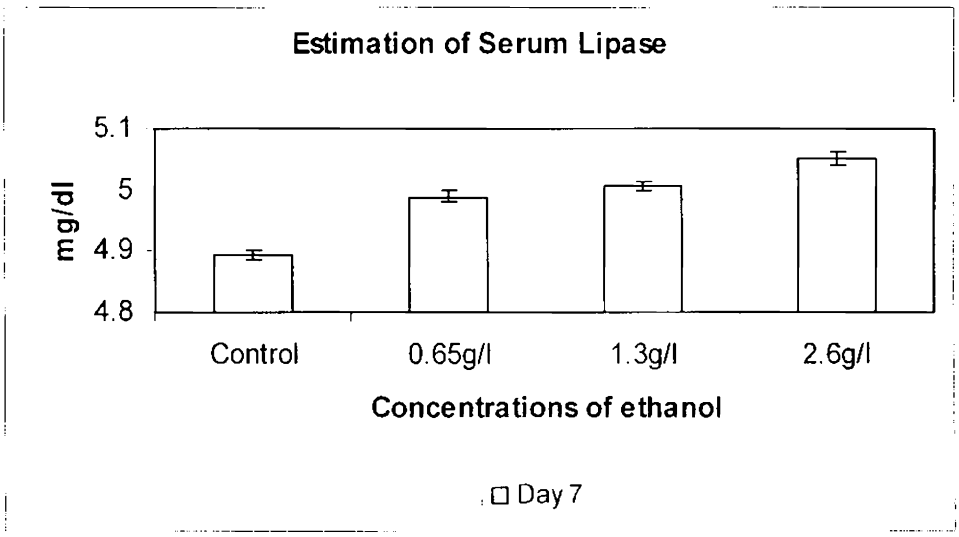
Table 7.4.15a ANOVA table for tissue free fatty acids

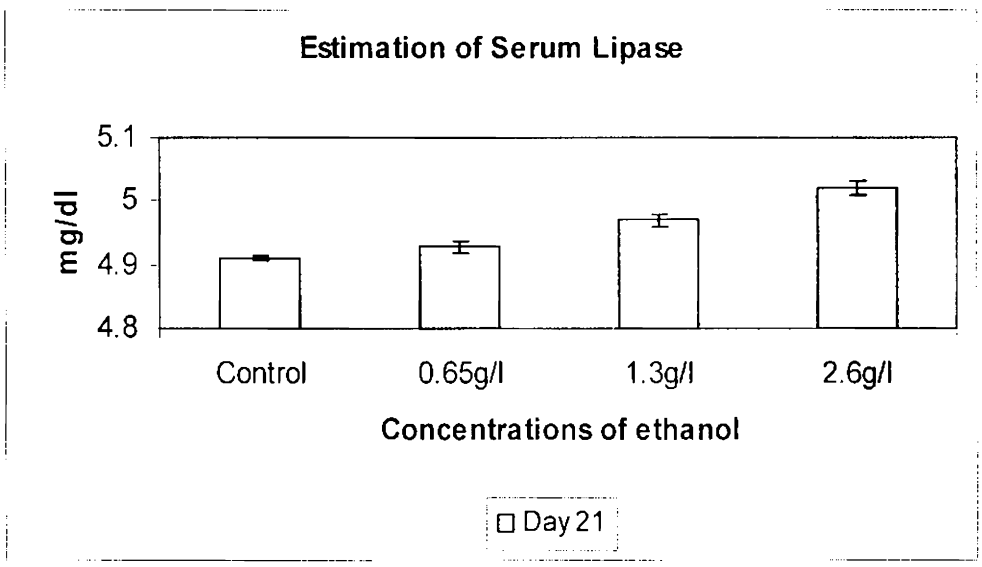
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	3.152	1	3.152	36.535	0.000
Between Concentrations	1.602	3	0.534	6.189	0.001
Between Tissues	99.917	1	99.917	1158.019	0.000
Error	7.765	90	0.086		
Total	112.436	95			

df-degrees of freedom

Statistical interpretation carried out by using Three Factor ANOVA exhibited that in the case of tissue free fatty acid levels, significant difference (P<0.001) was obtained when the effect between days of exposure, between concentrations and also between tissues were taken into account. This in turn indicates that effect of free fatty acids in tissues seems to be highly significant.

Figure 7.4.16 Levels of lipase activity in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Lipase activity in serum was found to be significantly increased ($P<0.001$) (Figure 7.4.16) when *O. mossambicus* was exposed for 7 and 21 days to various sub lethal ethanol concentrations with respect to control. Statistical analysis using ANOVA corroborated the above mentioned statement (Table 7.4.16a).

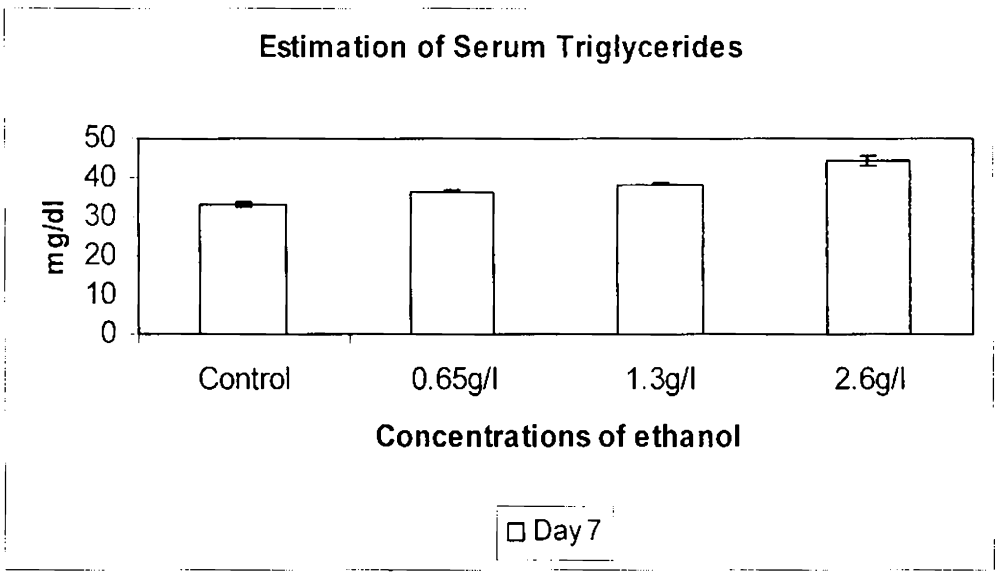
Table 7.4.16a ANOVA table for serum lipase

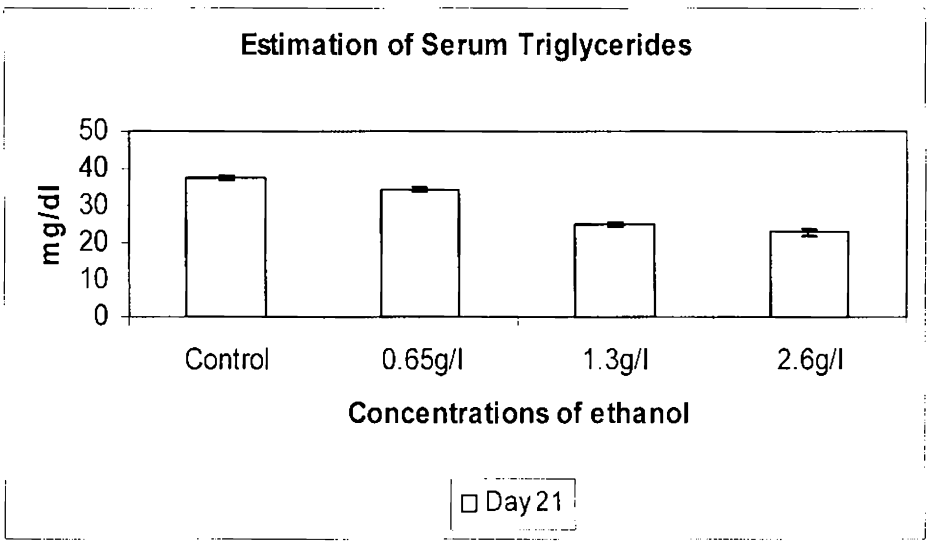
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	0.009	1	0.009	125.719	0.000
Between Concentrations	0.113	3	0.038	544.196	0.000
Days of Exposure × Concentration	0.010	3	0.003	46.293	0.000
Error	0.003	40	6.94E-005		
Total	0.135	47			

df-degrees of freedom

Statistical analysis done by using Two Factor ANOVA revealed that serum lipase levels varied significantly between days ($P<0.001$). There was significant difference between concentrations ($P<0.001$). When both the days as well as concentrations (interaction) were considered, significant difference ($P<0.001$) was observed.

Figure 7.4.17 Levels of triglycerides in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





From the graph (Figure 7.4.17) it can be concluded that serum triglyceride levels varied significantly (P<0.001) in *O. mossambicus* during immediate and prolonged exposure periods. Employing ANOVA justifies the above statement (Table 7.4.17a).

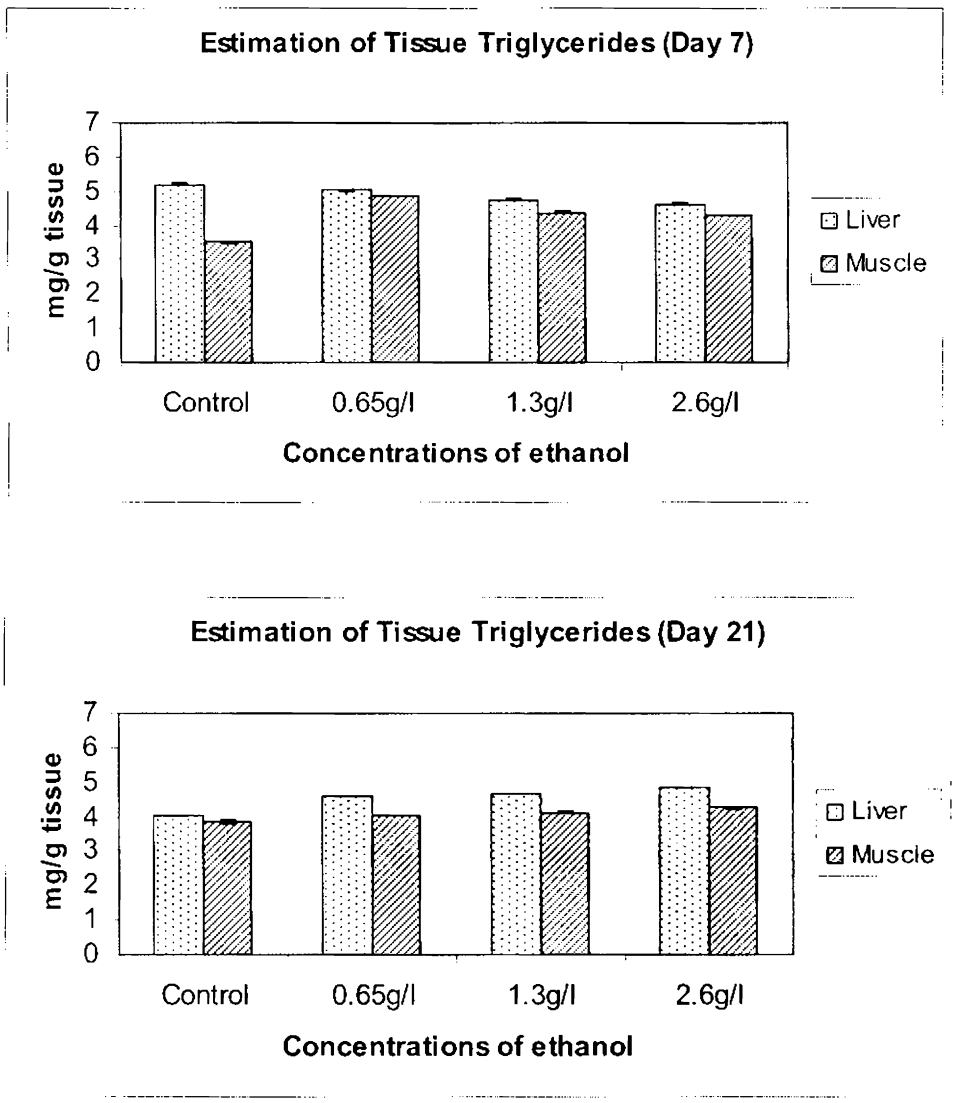
Table 7.4.17a ANOVA table for serum triglycerides

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	799.843	1	799.843	1402.064	0.000
Between Concentrations	114.781	3	38.260	67.067	0.000
Days of Exposure × Concentration	1169.582	3	389.861	683.396	0.000
Error	22.819	40	0.570		
Total	2107.025	47			

df-degrees of freedom

Two Factor ANOVA table revealed that in the case of serum triglyceride levels between days, significant difference (P<0.001) was observed. Between concentrations there was a significant difference (P<0.001). When taken into consideration the effects of both days as well as concentrations (Interaction), significant difference (P<0.001) was observed.

Figure 7.4.18 Levels of triglycerides in the tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



A significant difference ($P<0.001$) in tissue triglyceride level was observed in *O. mossambicus* when treated with various sub lethal concentrations of ethanol with respect to control during 7 and 21 days of exposure period (Figure 7.4.18).This was statistically supported by employing ANOVA and the results obtained is mentioned below (Table 7.4.18a).

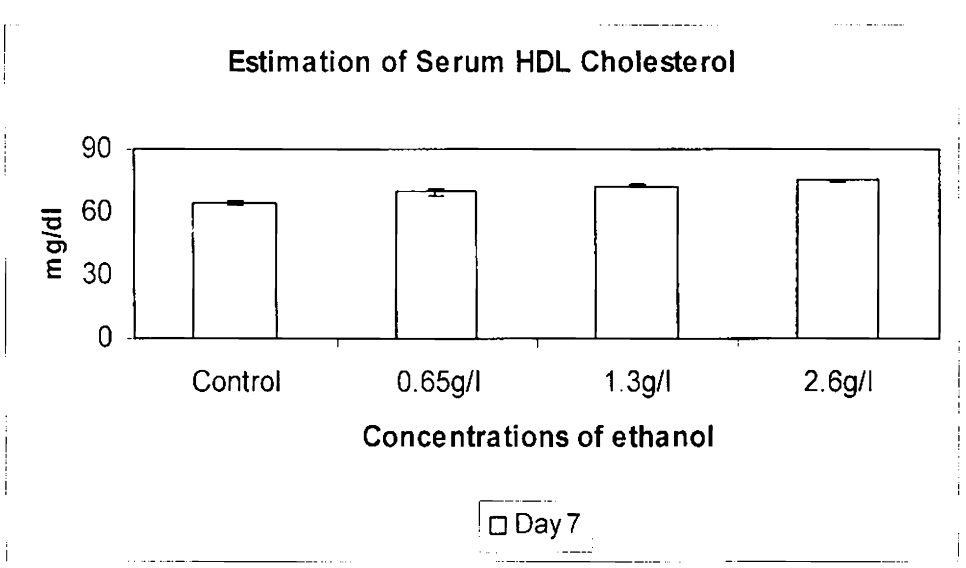
Table 7.4.18a ANOVA table for tissue triglycerides

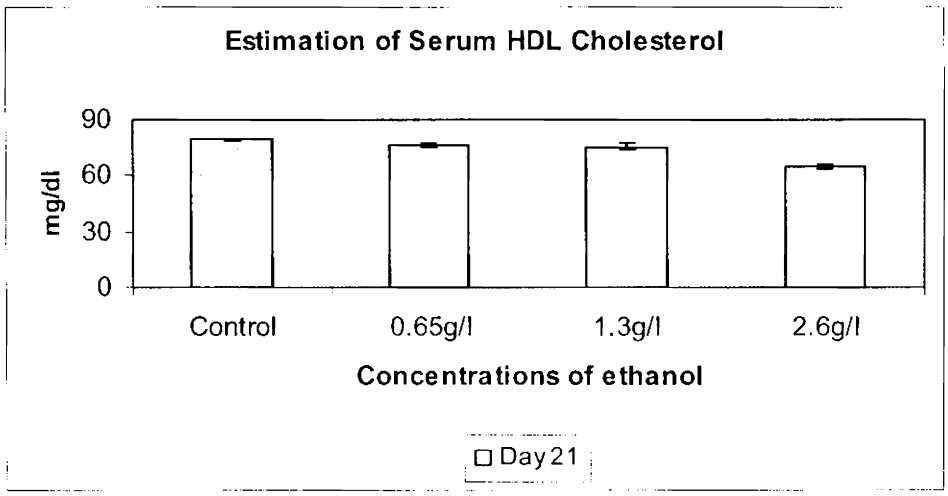
Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	1.720	1	1.720	23.246	0.000
Between Concentrations	3.001	3	1.000	13.520	0.000
Between Tissues	7.425	1	7.425	100.352	0.000
Error	6.659	90	0.074		
Total	18.805	95			

df-degrees of freedom

Analysis by Three Factor ANOVA table indicated that in the case of tissue triglycerides significant difference ($P<0.001$) was obtained when the effect between days of exposure, between concentrations and the effect between tissues were taken into account.

Figure 7.4.19 Levels of HDL cholesterol in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.





Highly significant (P<0.001) alterations were observed in the serum HDL cholesterol level in all the three treatment groups when compared to control group of *O. mossambicus* during 7 and 21 days of exposure period (Figure 7.4.19). Statistics using ANOVA was carried out which confirms the above statement and is depicted below (Table 7.4.19a).

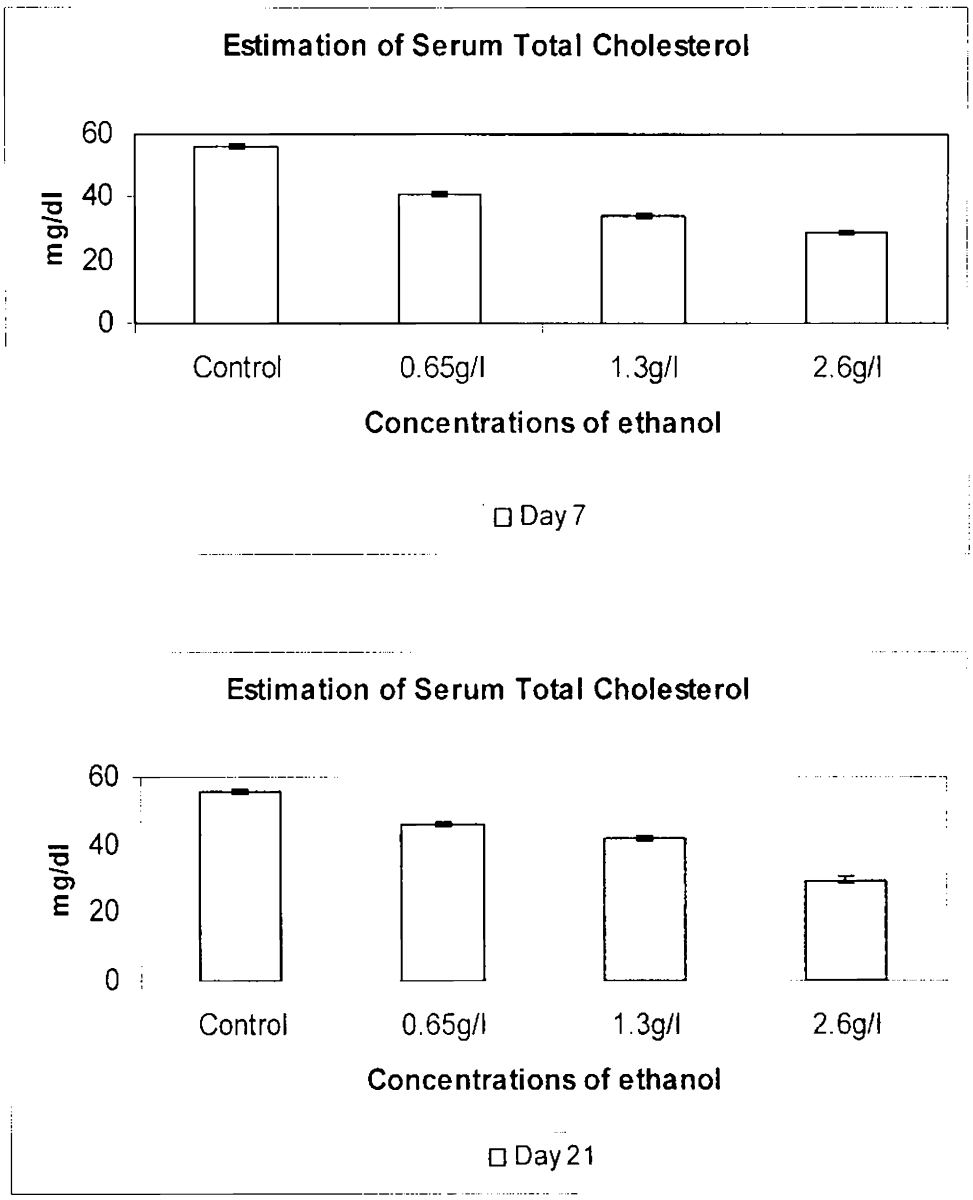
Table 7.4.19a ANOVA table for serum HDL cholesterol

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	161.553	1	161.553	129.293	0.000
Between Concentrations	97.747	3	32.582	26.076	0.000
Days of Exposure × Concentration	998.381	3	332.794	266.338	0.000
Error	49.981	40	1.250		
Total	1307.662	47			

df-degrees of freedom

By using Two Factor ANOVA it was noted that serum HDL Cholesterol levels varied significantly between days (P<0.001). Between concentrations a marked significant difference (P<0.001) was noted. When both the days as well as concentrations (Interaction), were considered significant difference (P<0.001) was observed.

Figure 7.4.20 Levels of total cholesterol in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



A significant ($P<0.001$) decrease in serum total cholesterol level (Figure 7.4.20) was noted in *O. mossambicus*, exposed to various sub lethal concentrations of ethanol than the control group. Statistical analysis conducted by using ANOVA substantiates this and the table is shown below (Table 7.4.20a).

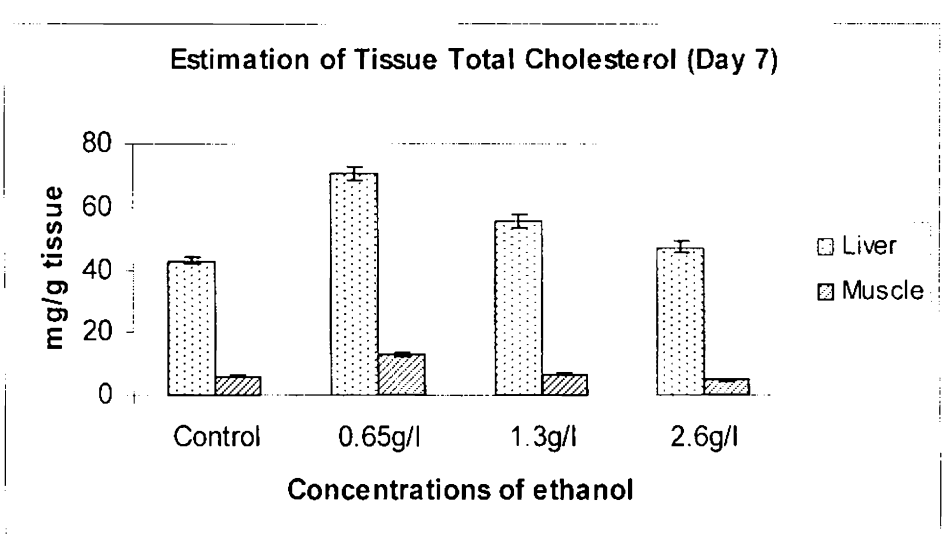
Table 7.4.20a ANOVA table for serum total cholesterol

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	130.812	1	130.812	221.684	0.000
Between Concentrations	4560.338	3	1520.113	2576.102	0.000
Days of Exposure × Concentration	127.268	3	42.423	71.893	0.000
Error	23.603	40	0.590		
Total	4842.021	47			

df-degrees of freedom

Studies done using Two Factor ANOVA indicated that serum cholesterol levels varied significantly between days ($P<0.001$). There was significant difference ($P<0.001$) between concentrations also. When both the days as well as concentrations (Interaction), were considered, then also significant difference ($P<0.001$) was noted.

Figure 7.4.21 Levels of total cholesterol in the tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



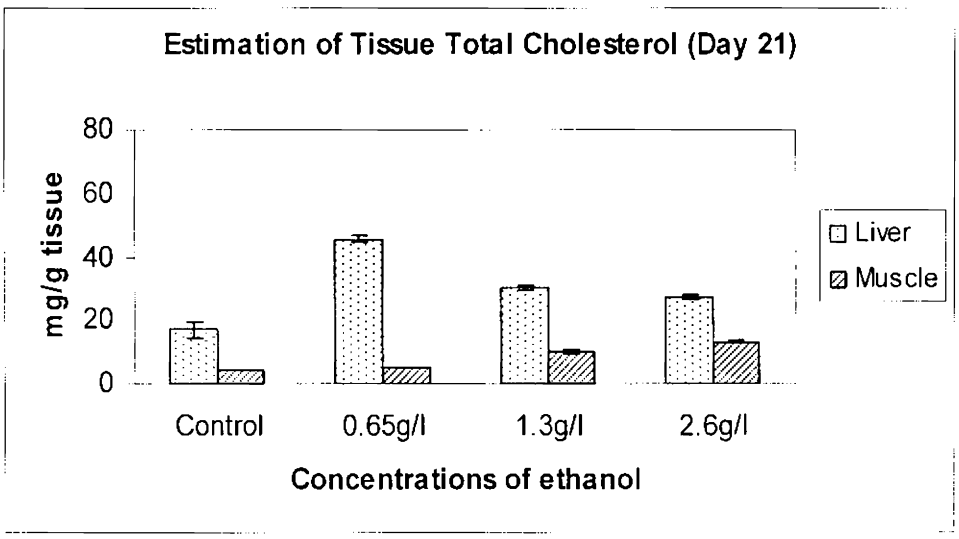


Figure 7.4.21 depicts significant changes (P<0.001) in the tissue total cholesterol levels in *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure period. Analysis carried out by applying ANOVA supports the above statement (Table 7.4.21a).

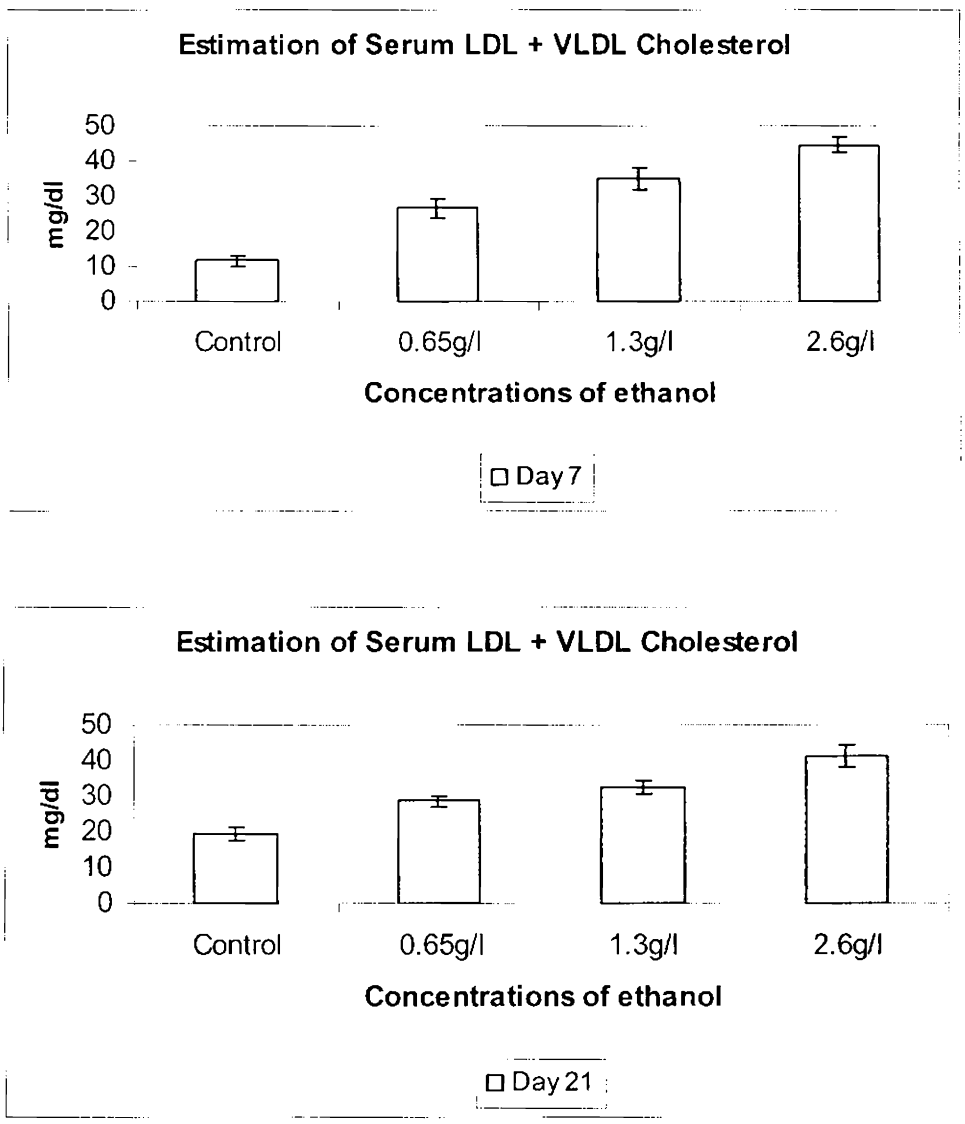
Table 7.4.21a ANOVA table for tissue total cholesterol

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	3243.375	1	3243.375	46.868	0.000
Between Concentrations	3237.567	3	1079.189	15.595	0.000
Between Tissues	27997.537	1	27997.537	404.577	0.000
Error	6228.185	90	69.202		
Total	40706.664	95			

df-degrees of freedom

Three Factor ANOVA table indicated that tissue total cholesterol levels varied significantly (P<0.001) between days of exposure. Between concentrations a marked significant difference (P<0.001) was noted. Between tissues also, significant difference (P<0.001) was observed.

Figure 7.4.22 Levels of LDL + VLDL Cholesterol in the serum of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Marked alterations were observed in the serum LDL+VLDL level of *O. mossambicus* exposed to 7 and 21 days (Figure 7.4.22). Statistical approach employing ANOVA corroborates this as can be inferred from the table below (Table 7.4.22a).

Table 7.4.22a ANOVA Table for serum LDL + VLDL Cholesterol

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Days of Exposure	9.765	1	9.765	1.738	0.195
Between Concentrations	4754.903	3	1584.968	282.102	0.000
Days of Exposure × Concentration	234.116	3	78.039	13.890	0.000
Error	224.737	40	5.618		
Total	5223.521	47			

df-degrees of freedom

By using Two Factor ANOVA table it was stated that serum LDL+ VLDL cholesterol levels exhibited no significant difference (NS) between days. Between concentrations a marked significant difference (P<0.001) was noted. After considering the interaction effects of both the days as well as concentrations significant difference (P<0.001) was seen.

Subsequent comparisons by multiple comparison test using Tukey followed by Dunnett's method is shown below (Table 7.4.23 and 7.4.24)

Table 7.4.23 Multiple Comparison Test (Serum)

	Groups	Total Lipids	Phospho lipids	Free fatty acids	Serum Lipase	TAG	HDL C	Total C	LDL+VLDL C
Dunnett	Control Vs 0.65g/l	0.010[b]	0.000[a]	0.000[a]	0.000[a]	0.999[d]	0.132[d]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.385[d]	0.000[a]	0.000[a]	0.001[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

TAG – Triglycerids, HDL-C - high density lipo protein cholesterol, Total C - Total Cholesterol,
LDL+VLDL C – Low density lipoprotein and very low density lipo protein cholesterol.

Table 7.4.24 Multiple Comparison Test (Tissues)

	Groups	Total Lipids	Phospho lipids	Free fatty acids	TAG	Total C
Tukey	Control Vs 0.65g/l	0.000[a]	0.001[a]	0.003[b]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.030[c]	0.002[b]	0.592[d]	0.000[a]	0.006[b]
	Control Vs 2.6g/l	0.710[d]	0.000[a]	0.991[d]	0.000[a]	0.094[d]
	0.65g/l Vs 1.3g/l	0.001[a]	0.993[d]	0.102[d]	0.233[d]	0.007[b]
	0.65g/l Vs 2.6g/l	0.000[a]	0.000[a]	0.001[a]	0.377[d]	0.000[a]
	1.3g/l Vs 2.6g/l	0.306[d]	0.000[a]	0.412[d]	0.991[d]	0.729[d]

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

TAG – Triglycerids, Total C - Total Cholesterol

A subsequent pair wise comparison in the case of serum sample between various concentrations with respect to control using Dunnett's method revealed that parameters such as phospholipids, lipase, total cholesterol and LDL+ VLDL cholesterol exhibited significant difference (P<0.001). In the case of serum total lipids in fishes exposed to ethanol concentration of 0.65 g/l when compared with control exhibited significant difference (P<0.01) whereas 1.3 g/l and 2.6 g/l conc of ethanol when compared with control exhibited significant difference (P<0.001). In the case of serum free fatty acids in fishes exposed to 1.3 g/l ethanol, on comparison with control exhibited no significant difference whereas those exposed to 0.65 g/l and 2.6 g/l ethanol exhibited significant difference (P<0.001). In the case of serum triglyceride levels and HDL Cholesterol levels, smallest concentration of ethanol failed to produce any significant difference, whereas the medium and high concentrations produced results with significant difference (P<0.001).

Subsequent pair wise comparison between various concentrations of liver and muscle tissues were carried out using Tukey which indicated that in the case of total lipids no significant difference (NS) was obtained when comparing 2.6 g/l ethanol exposed fishes with those exposed to 1.3 g/l ethanol and control ones;

whereas all other concentrations exhibited significant difference (P<0.001) when subjected to all possible interactions. In the case of phospholipids when 1.3 g/l was compared with 0.65 g/l no significant difference was seen whereas 1.3 g/l when being compared with control exhibited significant difference (P<0.01). In the case of free fatty acids 1.3 g/l when compared with control showed no significant difference (NS). Also 1.3 g/l when being compared with0.65 g/l and 2.6 g/l when being compared with 1.3 g/l also exhibited no significant difference (NS). In the case of triglycerides, 0.65 g/l when being compared with 1.3 g/l and 2.6 g/l exhibited no significant difference (NS) whereas 2.6 g/l when been compared with 1.3 g/l also showed the same pattern. In the case of total cholesterol 2.6 g/l when compared with control and 1.3 g/l when being compared with 2.6 g/l also showed no significant difference (NS).

7.4D Discussion

Remla *et al.* (1991) has reported that administration of ethanol to rats causes changes in the metabolism of serum and tissue lipids. HMG CoA reductase catalyses the rate limiting step in cholesterol biosynthesis and its activity directly correlates with the extent of tissue cholesterol synthesis which seems to be significantly activated during ethanol ingestion (Ashakumary and Vijayammal, 1993). HMG CoA Reductase activity was determined in the present study by a ratio method (HMG CoA /Mevalonate) in which lower ratio indicates higher activity. Activity of this enzyme showed a significant increase (P<0.001) (Fig 7.4.3) in all the tissues. Higher activity was indicated in the gills of fresh water teleost *O. mossambicus* when subjected to varying sub lethal concentrations of ethanol for 7 and 21 days. The above observation was supported by the findings of Ashakumary and Vijayammal (1993) who too obtained similar result in the rats when administered with alcohol and nicotine for a period of 3 months. Findings cited by Rajasree *et al.* (1999) too support the present study based on their observations on liver and intestine of rats treated with alcohol for 45 days. Thus it is logical to expect that any effect of ethanol on cholesterol biosynthesis will be reflected in the activities of HMG-CoA reductase. Therefore, it can be inferred that immediate as well as prolonged

exposure to ethanol brings about increased cholesterogenesis, which could be evidenced by the activity of HMG CoA reductase.

The interaction of ethanol with lipid metabolism is complex. Ethanol, a hydrophilic compound, is rapidly absorbed from the gastrointestinal tract and is uniformly distributed. The majority of the ethanol that enters the body is completely oxidized. It is well established that exposure to toxicants produces many biochemical changes in fish, which precede cellular systemic dysfunction. Ethanol is a powerful inducer of hyperlipidemia. It also causes a change in the metabolism of lipoproteins. Marked alterations in lipid metabolism have been reported in chronic conditions. Liver being the major site for detoxification is the primary target for environmental or occupational toxic exposure.

The concentration of total lipids present in the serum and tissues of *O. mossambicus* seems to be sensitive to ethanol which ultimately depends upon many factors such as the type of the contaminant, its concentration, mode of action, duration of exposure and type of fish species used. In the present study an increase (Figure 7.4.10) in the serum total lipid was observed when *O. mossambicus* was exposed to ethanol for 7 days. The observed increase in serum total lipid in the present study may be due to increased production by the liver and other tissues which resulted in the release of lipid constituents from the damaged cell membranes. Another possible reason for the increase in serum total lipid may be due to increased biogenesis of lipid resulting in hyperlipaemia. Increased synthesis or decreased lipid deposition or both resulted in simultaneous accumulation of lipids in the blood and in the liver as mentioned by Ashakumary and Vijayammal (1993). Similar increase in serum total lipid was cited by El-Elaimy *et al.* (1988) who also noticed marked increase in the total lipid in the blood of the fish inhabiting the polluted areas. Similar observations were noted by Wright *et al.* (1990) in rats exposed to ozone. The decrease in the serum total lipid (Figure 7.4.10) observed when *O. mossambicus* was exposed to varying sub lethal concentrations of ethanol for 21 days indicate the decrease in the production by the liver and other tissues which ultimately resulted in the decreased rate of the release of lipid constituents from damaged cell membranes. The changes observed in the serum lipids serve as sensitive indicators of the

progression of liver damage in alcoholics (Baraona and Lieber, 1979). In the present study an increase in total lipid in fishes exposed to 0.65g/l ethanol followed by a decrease at 1.3 g/l and 2.6 g/l ethanol was observed in the liver tissues of *O. mossambicus* when exposed to 7 days of ethanol. Elevated lipid contents observed at 0.65 g/l seems to be frequently associated with increased lipogenesis. The depletion in the hepatic total lipid observed at 1.3 g/l and 2.6 g/l could be due to their active mobilization towards the blood and/or tissue metabolism. Another possible reason for the decrease might be due to the utilization of lipid to meet the additional energy requirements when fishes are under stress. Reduction of lipid content in this study may have been due to the utilization of lipids for energy demand under stress condition. This statement was supported by Harpert *et al.* (1977). Similar observations were cited by Vijayamohanan and Achutan (2000) who too observed a decrease in the lipid content in the liver tissues of *O. mossambicus* and *E. maculatus* when compared with the controls on exposure to three sub lethal concentrations of titanium dioxide industrial effluent for 7 days. Findings stated by Kazufumi *et al.* (2003) also supports the present data. Lipid content in the muscle tissues of *O. mossambicus* exposed to different sub lethal concentrations of ethanol for 7 days exhibited a marked increasing trend. Similar trend was observed in fishes exposed to different concentrations of ethanol for 21 days ($P<0.001$) (Figure 7.4.11), in the liver and muscle tissues. The increase in lipid content in fish tissue might possibly be due to increased lipogenesis or less lipid mobilization. It can also be inferred that the increase in the amount of total lipid level in the liver tissue is mainly followed by the depletion in the level of glycogen and protein due to alcohol intoxication. An increase in total lipid level was found by Binduja *et al.* (1999) when rats were treated with ethyl alcohol for 45 days. The liver plays a central role in coordinating various metabolic functions of the body. Alcohol is an important cause of various liver diseases. The interaction of ethanol with lipid metabolism is complex. When ethanol is present, it becomes the preferred fuel for the liver and displaces fat as a source of energy. This blocks fat oxidation and favours fat accumulation (Leiber and Schmid, 1961). The accumulation of fat in the liver acts as a stimulus for the secretion of lipoproteins into the bloodstream and the development of hyperlipidemia.

A significant increase (P<0.001) (Fig 7.4.12a) in the serum and tissue phospholipid levels were observed when *O. mossambicus* was subjected to short term as well as prolonged exposure to ethanol. Phospholipid levels in muscle tissue recorded a significant decrease (Table 7.4.6) when fishes were exposed to 7 days at 2.6g/l ethanol. Alcohol facilitates the esterification of accumulated fatty acids to triglycerides, phospholipids, and cholesterol esters, all of which gets accumulated in the liver. Reports by Indira and Kurup (1982) support the present observations. Mahendran and Shyamala Devi (2001) also observed an increase in the concentration of phospholipids in the serum and liver tissues of rats when treated with ethanol for 45 days. Cholesterol and phospholipid levels were significantly higher in the alcohol-treated rats. The increase in the phospholipid level in the liver and muscle tissues observed in the present study might be due to non-utilization of this lipid by the tissues.

A significant increase (P< 0.001) (Fig 7.4.14a and Fig 7.4.15a) in the free fatty acid levels in the serum and muscle tissues were observed when *O. mossambicus* was exposed to ethanol for 7 days. In the case of liver tissue an increase was observed at ethanol concentration of 0.65 g/l followed by a decrease at 1.3 g/l and 2.6 g/l when *O. mossambicus* was exposed to this toxicant for 7 days. A possible reason for the increase in serum and muscle tissues may be due to any sort of unfavourable conditions (such as hard stress, intensive muscle load etc) which might have resulted in the increase of free fatty acid concentrations. The findings of Newsholm and Start (1973) support the above statement. Another possible reason for the increase of free fatty acid levels observed in the present study might be due to non-utilization of these lipids by the tissues. In the case of prolonged exposure a significant decrease was noted in the case of serum and muscle tissues followed by an increase in the liver tissues of *O. mossambicus* when exposed to ethanol for 21 days. Increase in the free fatty acids concentrations contribute to the accumulation of lipid in the liver which suggests that the free fatty acids were derived from the adipose tissue. It can also be stated that any condition of fish entering into a state of hypoxia or anoxia brings about a significant decline in the free fatty acid levels. Guido *et al.* (2002) have also reached the same conclusion based on their work in carp, goldfish and trout. A similar increase in the concentrations of free fatty acids

were observed in serum and various tissues when rats were administered with alcohol for 90 days as cited by Vijayammal and Ashakumary (1993). Indira and Kurup (1982) also support the present observations. Mahendran and Shyamala Devi (2001) also observed similar trend in the concentration of free fatty acids in the serum and liver tissues of rats when treated for 45 days with ethanol.

Serum lipase (triacylglycerol acylhydrolase, E.C 3.1.1.3) seems to be important in the diagnosis of acute pancreatitis (Lott and Lu, 1991; Lott *et al.*, 1986; Yang *et al.*, 2005). In the present study a significant increase ($P<0.001$) (Fig 7.4.16a) in serum lipase levels was observed when *O. mossambicus* was exposed to different sub lethal concentrations of ethanol for 7 and 21 days. The increase in serum lipase activity refers to injury of the pancreatic acinar cells which results in the leakage of this enzyme into the blood leading to hyperlipasemia. The present increase in the level of serum lipase activity was in turn supported by the finding of Atef (2007) who too observed similar increase in the serum lipase activity in *O. niloticus* when being intoxicated with nickel for 21 days.

As in mammals, fish triglycerides seem to be the central metabolites in lipid metabolism. Triglyceride concentration is important in evaluating lipid metabolism in which higher levels may occur with nephrotic syndrome and glycogen storage impairment. An increase in serum and muscle triglyceride value (Fig 7.4.7) was observed when the fish *O. mossambicus* was subjected to ethanol for 7 days. The rise in triglycerides serves as an indicator of altered fat metabolism occuring in the liver (Krajnovic and Krajnovic, 1992). Homeostasis of lipids is one of the principal liver functions, any change in the serum triglyceride concentration has been used as an indicator of liver dysfunction (Kaplan *et al.*, 1988). Muazzez *et al.* (2008) observed an increase in serum triglyceride when freshwater fish *O. niloticus* was subjected to metal exposure for 7 days. Similar reports were put forth by Abdelmeguid *et al.* (2002) who observed an increase in serum triglyceride level in *Tilapia zilii* collected from the most polluted locations of lake Maryut. Similarly there was a decrease in the triglyceride value in the liver tissues also. The decrease in TAG refers to lipolysis occurring in the fish tissues. As a result of complex lipid disassimilations, the fish spends a large amount of energy to combat the stress. In

the present study a decrease (Fig 7.4.17) in serum triglyceride value obtained when *O. mossambicus* was exposed to ethanol for 21 days refers to the increase in the activity of lipase enzyme which could probably result in the increased uptake of circulating triglycerides leading to lowering of serum triglyceride level under chronic conditions. Lipoprotein lipase is the enzyme involved in the uptake of circulating triglyceride rich lipoproteins in alcohol. Similarly a decrease in serum triglyceride value was observed when freshwater fish *O. niloticus* was subjected to metal exposure for 21 days (Muazzez *et al.* 2008). The increase (Figure 7.4.3.18a) in the liver and muscle triglyceride value when *O. mossambicus* was exposed for 21 days refers to the enhanced endogenous synthesis of triglycerides which reduces the utilization of dietary lipids resulting in their accumulation in the liver. Another possible reason could be the damage brought about to the liver cells indicating impaired liver function. Ethanol increases triglyceride levels thus indicating imbalance in lipid metabolism in liver and muscle tissues. As per Mahendran and Shyamala Devi (2001), chronic administration of ethanol was found to produce an accumulation of triglycerides in the liver. The increased level of triglycerides seen in alcohol fed rats may be due to increased biosynthesis. Increased level of triglycerides was found in the muscle and liver of fish collected from lake Qarun when exposed to heavy metals and pesticides. The elevation in the levels of tissue triglycerides may be attributed to enhanced triglyceride synthesis or to reduced triglyceride catabolism as indicated by Pant and Singh (1983). The increase in these energy reserves in response to pollution could be due to the fact that excess energy reserves (as glucose, triglycerides and cholesterol)are required by organisms to mediate the effects of stress (Shulman, 1974; Lee *et al.*, 1983) and to serve as energy buffers during periods of harsh environmental conditions and food shortages (Adams and McLean, 1985).

HDL-Cholesterol is mainly involved in the transfer of cholesterol from tissues to the liver, for degradation. There are several reports regarding a positive correlation between HDL-Cholesterol and alcohol intake. An increase (Fig 7.4.7) in the HDL- Cholesterol level in the serum and muscle tissues of *O. mossambicus* exposed to 7 days refers to the increased lipoprotein lipase activity which results in the enhanced transfer of surface components (such as cholesterol and phospholipids)

from triglyceride rich lipoproteins to HDL which mainly arises during lipolysis. Another possible explanation of increased HDL-C can be due to ethanol stimulated secretion of apolipoprotein A-1 (apo A-1) by hepatocytes which upregulates lipoprotein lipase and apoA-1 at the level of gene expression.

A decrease in the HDL cholesterol value was obtained in the liver tissues of *O. mossambicus* when exposed to 7 days which is in agreement with the increased concentrations of cholesterol present in *O. mossambicus*. The observations made by Bindu and Annamalai (2003) in the liver tissues of rats when treated with ethanol supports the present finding. In the present study a significant decrease ($P<0.001$) in the HDL Cholesterol in the serum value was observed in *O. mossambicus* when exposed to 21 days which in turn was supported by Bindu and Annamalai (2003). Similar findings were cited by Indira *et al.* (2001) based on their study on rats treated with ethanol under chronic exposure. A significant increase ($P<0.001$) in the HDL Cholesterol value in both liver and muscle tissues was obtained when *O. mossambicus* was exposed to ethanol for 21 days which usually refers to decreased concentrations of cholesterol present in the tissues of *O. mossambicus*.

VLDL which is primarily formed in liver gets converted to intermediate density lipo-protein (IDL) and LDL. LDL gets attached to the receptor on the surface of many cells in the body and is taken into cells by endocytosis. A significant increase ($P<0.001$) in the LDL+ VLDL values was observed in the serum of *O. mossambicus* when exposed to different sub lethal concentrations of ethanol for 7 and 21 days. Indira *et al.* (2001) also noticed similar increase in LDL+ VLDL cholesterol values in rats when treated with ethanol for 15 days. Increased level of LDL+VLDL cholesterol seems to be consistent with the previous report of Fricker *et al.* (1990). Bindu and Annamalai (2003) also observed increase in LDL values in the serum of rats on treatment with ethanol for 30 days.

The concentration of total cholesterol present in the serum and tissues of *O. mossambicus* seems to be very much sensitive to ethanol. The marked alterations observed in the present study seems to be dependent on many factors such as the concentration of ethanol, its duration of exposure etc. The decrease in the serum cholesterol value observed during short term and prolonged exposure to ethanol may

be due to decrease in the production by the liver and muscle tissues. This may bring about decrease in the release of cholesterol and other lipid constituents from damaged cell membranes. Decrease in the level of serum cholesterol is indicative of liver dysfunction. Another reason could be the decrease in the hepatic excretion of cholesterol. It can also be commented that decrease in serum cholesterol may be due to the increased activity of plasma lecithin cholesterol acyl transferase. Ashakumary and Vijayammal (1993) seems to contradict the present findings by reporting an increase in serum cholesterol level with a concomitant decrese in PLCAT enzyme. A significant decrease ($P<0.001$) in the serum cholesterol value was cited when *O. mossambicus* was subjected to different sub lethal concentrations of ethanol for 7 and 21 days.

In the present study an increase in liver cholesterol value was observed when *O. mossambicus* was exposed to ethanol for 7 days. In the case of muscle tissue an increase in cholesterol value was observed in those exposed to ethanol concentration of 0.65 g/l and 1.3 g/l; followed by a decrease at 2.6 g/l. The elevation of the tissue cholesterol level may be attributed to enhanced cholesterol synthesis or due to reduced cholesterol catabolism. An increased level of cholesterol was found in the muscle and liver of fish collected from lake Qarun when exposed to heavy metals and pesticides as mentioned by Pant and Singh (1983). Chronic alcohol intake is known to produce hypercholesterolemia, hyperlipidemia and hypertriglyceridemia. In chronic lipid accumulation the liver cells become fibrotic and leads to impaired liver function. Ethanol thus increases triglycerides and cholesterol levels thus inducing an imbalance in lipid metabolism in liver and muscle. In the case of tissue cholesterol value, when liver and muscle tissues were taken into consideration a marked significant increase ($P<0.001$) was noted when *O. mossambicus* was exposed to ethanol for 21 days. The reason for the increase in cholesterol levels in tissues such as liver and muscle was mainly due to the enhanced cholesterol production by the liver resulting in significant increase in tissue cholesterol of ethanol exposed fish. An increase in liver and muscle cholesterol level was observed when *O. mossambicus* was subjected to three different sub lethal concentrations of ethanol for 21 days. This in turn was supported by the findings stated by Balasubramaniam *et al.* (1999) who also observed an increase in liver and muscle

cholesterol level when *O. mossambicus* (Trewaves) were exposed to sub lethal concentration of urea for 20 days.

The present study revealed that *O. mossambicus* is sensitive to sub lethal concentrations of ethanol. In addition to changes in the serum parameter levels changes were also exhibited in the biochemical composition of tissues such as liver and muscle which indicates that changes brought about in the lipid profile reflect changes in the normal activities of various functional systems. It can be concluded that biochemical parameters that fluctuated in response to deleterious effects of pollution could be ranked as possible biomarkers of pollution.

Chapter 8

EFFECT OF ETHANOL ON LYSOSOMAL MEMBRANE STABILITY (*In vitro* and *In vivo* Conditions)

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8.1A Introduction

Cell represents the smallest unit which integrates all functions of life such as metabolism, growth, movement, sensitivity and reproduction. Studies on the interaction between life and xenobiotics ultimately focus on the cell. This consideration lies on the basic concept of ecotoxicology. The xenobiotic-induced sub lethal cellular pathology reflects perturbations of function and structure at the molecular level. Recently, many authors have outlined the importance of environmental impact assessment programmes including methods which measure the biological effects of pollutants on the health condition of organisms (Moore, 1985). Many studies have been carried out to develop stress indices at different levels of biological organization.

Ethanol being a small molecule is freely permeable across membranes. Thus, ethanol induced changes in the nature of the aqueous environment affect cytoplasmic enzymes and functions in addition to those on the cell surface and within the membrane. Ethanol decreases the energy barrier by its effects both on the aqueous environment (decreasing polarity) and on the hydrophobic core (increasing polarity), which increases the permeability of the membrane to polar and charged molecules.

Lysosomes are highly conserved multi-functional cellular organelles present in almost all cells of eukaryotic organisms ranging from yeast to humans. They mainly comprise of a heterogeneous group of cytoplasmic organelles that mediate the digestive and lytic processes of the cell (Weismann, 1965). They are mainly bound by a semi permeable lipoprotein membrane and contain a battery of over sixty hydrolytic enzymes such as acid phosphatase (ACP), β glucuronidase, cathepsin, aryl sulfatase etc. They play a very important role in breaking down the substances within a cell (autophagy) or substances that have been taken in from outside the cell (heterophagy) (Bozzola and Russell, 1992; Holtzman, 1976).

Lysosomal reactions appear to provide useful biomarkers that are diagnostic for cell injury and serve as putative indicators for further pathology (Moore, 1990). Lysosomal membrane stability is referred to as a predictive indicator for cell injury and pathology. Lysosomal stability may be useful as an early warning signal which indicates exposure to and effects of environmental pollutants present in the aquatic ecosystem. It has been shown that lysosome targeted stress results in permeabilisation of lysosomal membrane, resulting in the relocation of lysosomal constituents to the cytosol and finally, apoptosis. Lysosome integrity serves as a simple and cost effective approach to study pollutant exposure and its effects which in turn can lead to membrane damage resulting in the leakage of their resident acid hydrolases into the cytosol thus damaging the cells. Disturbed lysosomal integrity was additionally assessed by estimation of the amount of acid phosphatase and β glucuronidase being translocated to the cytosol which mainly arises due to lysosomal stress. The release of typical lysosomal enzymes β-glucuronidase and acid phosphatase from the lysosome rich fraction of the liver has been taken as a

measure of lysosomal stability. The lysosomal stability was measured in terms of the lysosomal enzyme release assay. In this chapter effort an has been made to study the lysosomal membrane stability in *in vitro* and *in vivo* conditions.

8.2B Materials and Methods

Collection, maintenance, acclimatization of fish, bioassay method and experimental design for ethanol based study were the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

8.2B.1 Preparation of tissue samples for experimental studies

(i) *In vivo* Studies

For conducting *in vivo* studies 2% liver homogenate was taken. Hepatic tissues were isolated from control fish and from those exposed to ethanol for 21 days followed by a periodical sampling at 7 days also. They were then homogenized separately in isotonic sucrose (0.33M).

(ii) *In vitro* Studies

For conducting *in vitro* studies 5% of liver homogenate was taken. Normal liver tissues were treated with three varying sub lethal concentrations of ethanol in such a way in which the final ethanol concentrations in the tubes were 0.65g/l,1.3g/l and 2.6g/l respectively. Apart from this, hepatic tissues from control fish were also taken which to serves as the control.

8.2B.1a Activity of lysosomal enzymes (β-glucuronidase (E.C 3.2.1.31) and acid phosphatase (E.C 3.1.3.2)) in the various sub cellular fractions of liver tissue of *O. mossambicus* (*in vitro* and *in vivo* conditions).

The homogenate obtained was centrifuged at 600g for 10 min in a high speed refrigerated centrifuge. The sediment comprising of nuclei, unbroken cells and plasma membrane (nuclear fraction) was separated. The supernatant was again centrifuged at 15000g for 30 min. The 15000g sediment (lysosomal rich fraction) and nuclear fraction were resuspended in citrate buffer of pH 4.8 containing 0.2%

Brij-35. The 15000g supernatant comprising of soluble fraction was diluted with an equal volume of double strength citrate buffer of pH 4.8. The activity of β-glucuronidase and acid phosphatase, were determined in all these three fractions (Plummer, 1987). The release of typical lysosomal enzymes glucuronidase and acid phosphatase from the lysosomes rich fraction of the liver has been taken as a measure of lysosomal stability.

8.2B.1b Rate of release of β glucuronidase from the lysosomal-rich fraction of liver or lysosomal enzyme release assay

(i) *In vivo* Studies

Hepatic tissue from control fish and from those exposed to three sub lethal concentrations of ethanol for 7 and 21 days were homogenized separately in isotonic sucrose and centrifuged to obtain the lysosomal fraction as mentioned above. The lysosomal pellet was washed, centrifuged at 15000g for 10 min and again resuspended in cold 0.33 M sucrose. A definite volume of this suspension was incubated at room temperature and aliquots were withdrawn at various time intervals of 0, 15, 30 and 45 minutes. The retrieved fractions were stored immediately at 0^0C. Both the control and test aliquots were centrifuged at 15000 g for 30 min to separate the unbroken lysosomes and glucuronidase activity released into the supernatant was determined.

8.2B.1c Methods used for the biochemical analysis

The following are the parameters studied under lysosomal membrane stability

a. **Estimation of acid phosphatase activity (E.C 3.1.3.2) was carried out as mentioned in chapter 7 Section 7.3.B.2 h.**
b. **Estimation of β-glucuronidase (E.C 3.2.1.31)**

Principle

β-glucuronidase activity was estimated by the method of Kawai and Anno (1971). This method involves the liberation of p- nitrophenol.

p-nitrophenyl β-D glucuronide+ H_20 $\xrightarrow{\beta - \text{Glucuronidase}}$ D Glucuronate+ p-nitro phenol

The liberated p-nitro phenol was measured at 400 nm.

Reagents:

1. Substrate: Dissolved 1 mg of p-nitro phenyl β-D glucuronide in 1.0 ml of distilled water.

2. 0.1 M acetate buffer of pH 5.4

Solution A: 0.2 M acetic acid (11.55 ml of glacial acetic acid was made upto one litre with distilled water).

Solution B: 0.2 M Sodium acetate (16.4 g of $C_2H_3O_2Na$ was made upto one litre with distilled water).

Mixed 28 ml of solution A and 22 ml of solution B. The pH was adjusted to 5.4. It was then made upto 100 ml with distilled water. Diluted 1 part of 0.2 M solution with 1 part of water to get 0.1 M solution.

1. Standard: 5.0 mg of p-nitrophenol was made upto 100 ml with distilled water.
2. Glycine buffer, 0.2 M, pH 10.7
3. Mixed equal volumes of 0.2 M glycine, 0.125 M sodium carbonate and 0.1 M sodium chloride.

Procedure

To 0.05 ml of substrate, 0.05 ml of acetate buffer was added and incubated with the sample at 37^0C for 1 hr. The reaction was arrested by the addition of 3.9 ml of glycine buffer (pH 10.7). Standards in the concentration range of 5-20 μg were also run simultaneously. The colour developed was then read at 400 nm. The enzyme activity obtained was expressed as units/protein in tissues.

c. Estimation of tissue protein was carried out as mentioned in chapter 4, section 4.1B. 2a.

8.3C Results

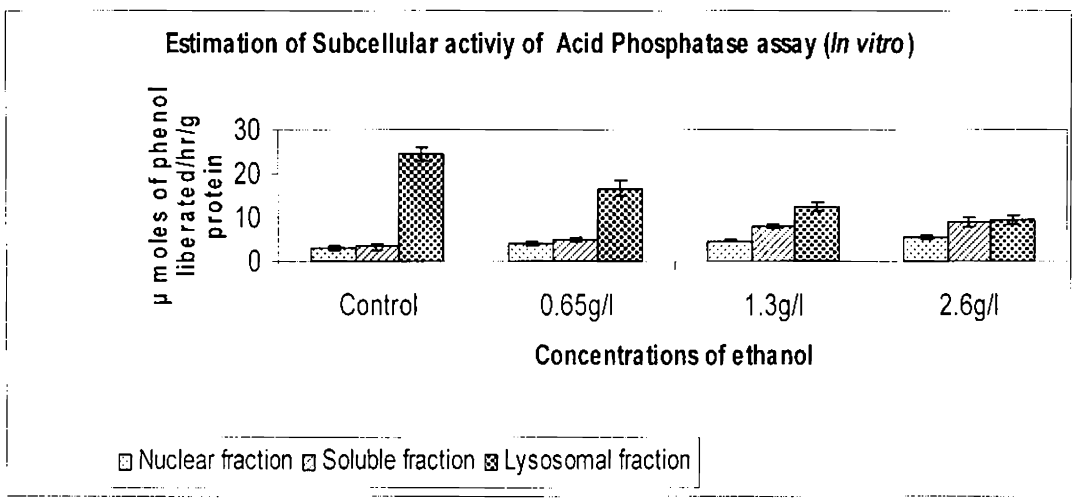
Effect of 0.65 g/l, 1.3 g/l and 2.6 g/l sub lethal concentrations of ethanol for 21 days followed by a periodical sampling at 7 days exhibited marked alterations on the sub cellular activity of lysosomal enzymes viz. β glucuronidase and acid phosphatase which is given in tables 8.3.1 to 8.3.14 and in figures 8.3.2 to 8.3.13. Results obtained were statistically analyzed by ANOVA (Analysis of Variance) followed by Dunnett's method.

Table 8.3.1 Effect of different concentrations of ethanol on the sub cellular activity of acid phosphatase in *O. mossambicus (In vitro)*

Sub cellular Activity	Control	Concentrations of ethanol		
		0.65g/l	1.3g/l	2.6g/l
Nuclear	3.027± 0.6870	3.962± 0.5326	4.523± 0.2340	5.310± 0.4516
Soluble	3.300± 0.7475	4.937± 0.5841	7.983± 0.6924	8.932± 1.0829
Lysosomal	24.56± 1.5165	16.71± 1.6015	12.69± 0.9785	9.545± 0.9017
Ratio Lysosomal to Soluble activity	7.731± 1.5847	3.414± 0.4096	1.596± 0.1604	1.083± 0.1846

Average of six values in each group ± SD of six observations
The values are expressed as μmoles of phenol liberated / hour/ gram protein.

Figure 8.3.2 Levels of sub cellular acid phosphatase activity in the hepatic tissue of *O. mossambicus (In- vitro)*



Highly significant ($P<0.001$) alterations were observed in all the three sub cellular fractions (nuclear, soluble and lysosomal) of the treatment groups when compared to control group of *O. mossambicus* during *in-vitro* studies (Figure 8.3.2). Statistics using ANOVA was carried out which confirms the above statement and is depicted below (Table 8.3.2a to 8.3.2c).

Table 8.3.2a ANOVA table for nuclear acid phosphatase activity *(In- vitro)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	16.620	3	5.540	21.850	0.000
Error	5.071	20	0.254		
Total	21.691	23			

df – degrees of freedom

Table 8.3.2b ANOVA table for soluble acid phosphatase activity *(In- vitro)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	123.704	3	41.235	64.632	0.000
Error	12.760	20	0.638		
Total	136.464	23			

df – degrees of freedom

Table 8.3.2c ANOVA table for lysosomal acid phosphatase activity *(In- vitro)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	758.166	3	252.722	152.360	0.000
Error	33.174	20	1.659		
Total	791.340	23			

df – degrees of freedom

Studies done by using one factor ANOVA indicated that there was an overall significant difference (P<0.001) in the acid phosphatase activity in the nuclear (Table 8.3.2a), soluble (Table 8.3.2b) and lysosomal (Table 8.3.2c) fractions of the different sub lethal concentrations of ethanol treated experimental animals when compared with control.

Table 8.3.3 Multiple comparison test (concentration) *(In- vitro)*

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method and the results are depicted in Table 8.3.3.

	Groups	Nuclear fraction	Soluble fraction	Lysosomal fraction
Dunnett	Control Vs 0.65g/l	0.012^{c}	0.006^{b}	0.000^{a}
	Control Vs 1.3g/l	0.000^{a}	0.000^{a}	0.000^{a}
	Control Vs 2.6g/l	0.000^{a}	0.000^{a}	0.000^{a}

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

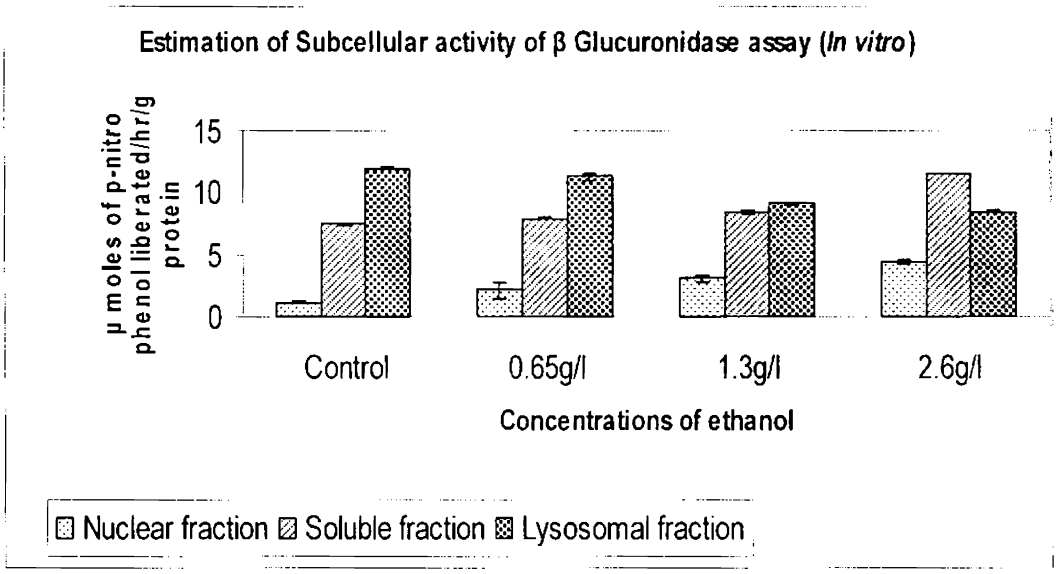
There was no significant difference (NS) in the acid phosphatase activity in the nuclear fraction of 0.65 g/l ethanol treated *O. mossambicus* when compared to control. In the case of 1.3 g/l and 2.6 g/l significant difference (P<0.001) was obtained when being compared with control. While considering the soluble fraction of acid phosphatase activity significant difference (P<0.01) was obtained at 0.65 g/l when compared with control, whereas 1.3 g/l and 2.6 g/l exhibited significant difference (P<0.001) with respect to control. Considering the acid phosphatase activity in the lysosomal fraction, significant difference (P<0.001) was observed at 0.65 g/l, 1.3 g/l and 2.6 g/l when compared with control.

Table 8.3.4 Effect of different concentrations of ethanol on the sub cellular activity of β-glucuronidase in *O. mossambicus (In- vitro)*

	Concentrations of ethanol			
Sub cellular Activity	**Control**	**0.65g/l**	**1.3g/l**	**2.6g/l**
Nuclear	1.182± 0.0462	2.118± 0.5973	3.040± 0.2888	4.337± 0.1700
Soluble	7.417± 0.0288	7.892± 0.1065	8.383± 0.1786	11.57± 0.0436
Lysosomal	11.97± 0.1515	11.26± 0.2740	9.093± 0.0809	8.480± 0.0346
Ratio Lysosomal to Soluble activity	1.614± 0.0164	1.427± 0.0415	1.085± 0.0295	0.733± 0.0040

Average of six values in each group ± SD of six observations
The values are expressed as μ moles of p-nitro phenol liberated/ hour/ gram protein.

Figure 8.3.5 Levels of sub cellular β - glucuronidase activity in the hepatic tissue of *O. mossambicus (In- vitro)*



From the graph (Figure 8.3.5) it can be concluded that β-glucuronidase activities varied significantly ($P<0.001$) in *O. mossambicus* in *in-vitro* studies. Employing ANOVA justifies the above statement (Table 8.3.5a to 8.3.5c).

Table 8.3.5a ANOVA table for nuclear β-glucuronidase activity *(In- vitro)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	32.605	3	10.868	92.257	0.000
Error	2.356	20	0.118		
Total	34.961	23			

df – degrees of freedom

Table 8.3.5b ANOVA table for soluble β-glucuronidase activity *(In- vitro)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	63.561	3	21.187	1843.671	0.000
Error	0.230	20	0.011		
Total	63.791	23			

df – degrees of freedom

Table 8.3.5c ANOVA table for lysosomal β-glucuronidase activity *(In- vitro)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	50.730	3	16.910	639.617	0.000
Error	0.529	20	0.026		
Total	51.259	23			

df – degrees of freedom

Statistical analysis by one factor ANOVA revealed that β–glucuronidase levels varied significantly ($P<0.001$) between concentrations in the nuclear (Table 8.3.5a), soluble (Table 8.3.5b) and lysosomal (Table 8.3.5c) fractions of the different sub lethal concentrations of ethanol treated experimental animals when compared with control.

Table 8.3.6 Multiple Comparison Test (Concentration) (*In- vitro*)

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method and the results are depicted in table 8.3.6.

	Groups	Nuclear fraction	Soluble fraction	Lysosomal fraction
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]

The values are significant at a=P < 0.001.

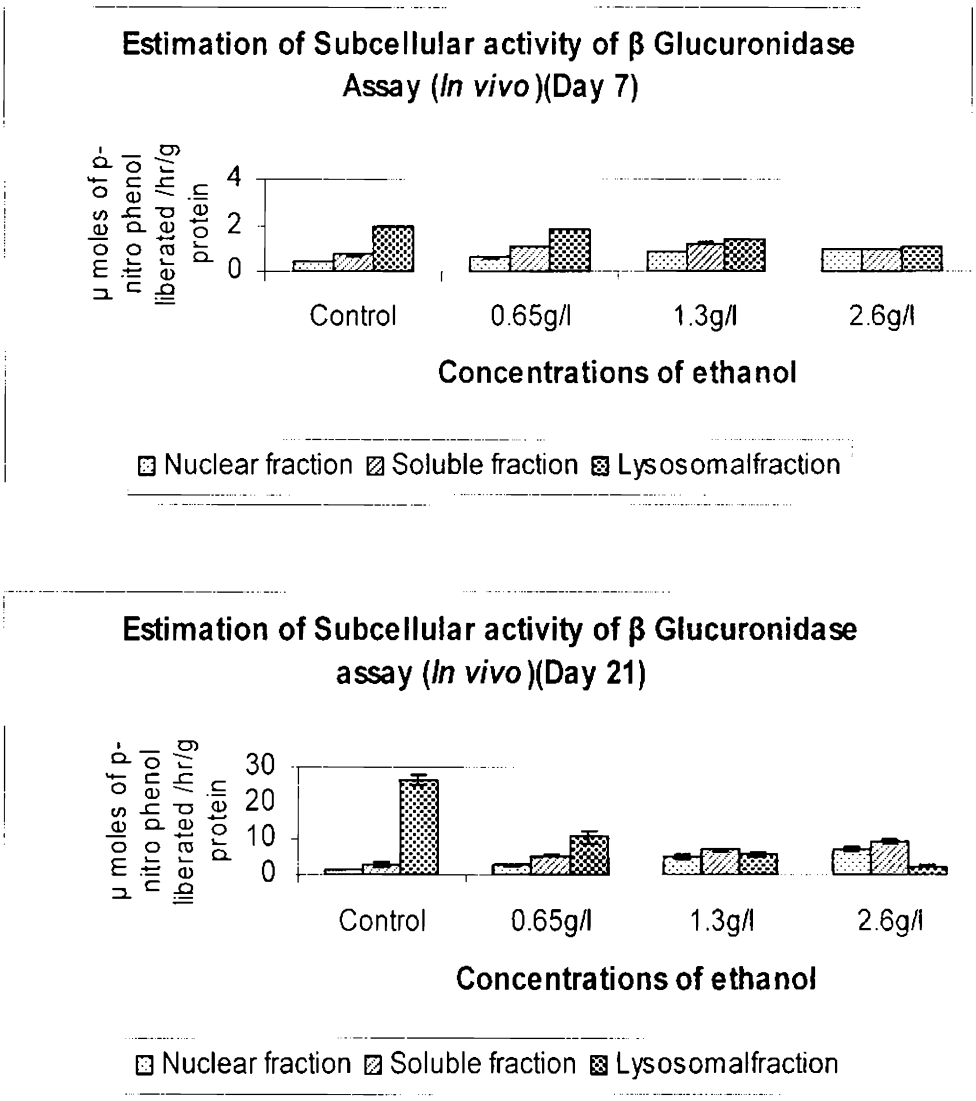
Pair wise comparison between various concentrations with respect to control indicated that ethanol at all the three sub lethal concentrations exhibited significant difference (P<0.001) in nuclear, soluble and lysosomal fractions when compared with control.

Table 8.3.7 Effect of different concentrations of ethanol on the sub cellular activity of β-glucuronidase in *O. mossambicus (In- vivo)*

	Sub cellular fractions	Control	Concentrations of ethanol 0.65g/l	1.3g/l	2.6g/l
Day 7	Nuclear	0.408± 0.0193	0.619± 0.0553	0.852± 0.0240	0.947± 0.0173
	Soluble	0.741± 0.0555	1.071± 0.0197	1.234± 0.0563	0.984± 0.0042
	Lysosomal	1.955± 0.0104	1.827± 0.0218	1.371± 0.0146	1.072± 0.0420
	Ratio Lysosomal to soluble activity	2.653± 0.2115	1.705± 0.0294	1.113± 0.0440	1.089± 0.0434
Day 21	Nuclear	1.403± 0.2830	2.558± 0.1498	5.002± 0.8025	7.362± 0.6568
	Soluble	3.059± 0.6071	5.112± 0.3739	7.000± 0.4825	9.126± 0.7639
	Lysosomal	26.53± 1.1909	10.66± 1.7620	5.867± 0.6144	2.461± 0.5867
	Ratio Lysosomal to soluble activity	8.971± 1.8451	2.1066± 0.4385	0.839± 0.0805	0.270± 0.0585

Average of six values in each group ± SD of six observations
The values are expressed as μ moles of p-nitro phenol liberated /hour/ gram protein.

Fig 8.3.8 Levels of sub cellular β-glucuronidase activity in the hepatic tissue of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol *(In- vivo)*



O. mossambicus exhibited marked significant ($P<0.001$) alterations in the β- glucuronidase activities in all the three sub cellular fractions when subjected to various sub lethal concentrations of ethanol (Figure 8.3.8). Investigations using ANOVA substantiates the above statement which is shown below (Table 8.3.8a to 8.3.8c).

Table 8.3.8a ANOVA table for nuclear β-glucuronidase activity *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	74.987	3	24.996	169.151	0.000
Between Days of Exposure	136.661	1	136.661	924.805	0.000
Concentration × Days of Exposure	52.715	3	17.572	118.911	0.000
Error	5.911	40	0.148		
Total	270.274	47			

df – degrees of freedom

Table 8.3.8b ANOVA table for soluble β-glucuronidase activity *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	66.232	3	22.077	132.656	0.000
Between Days of Exposure	308.037	1	308.037	1850.897	0.000
Concentrations × Days of Exposure	55.666	3	18.555	111.492	0.000
Error	6.657	40	0.166		
Total	436.592	47			

df – degrees of freedom

Table 8.3.8c ANOVA table for lysosomal β-glucuronidase activity *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	1088.063	3	362.688	552.963	0.000
Between Days of Exposure	1157.925	1	1157.925	1765.405	0.000
Concentrations × Days of Exposure	953.861	3	317.954	484.761	0.000
Error	26.236	40	0.656		
Total	3226.085	47			

df – degrees of freedom

By using Two factor ANOVA it was noted that β-glucuronidase levels exhibited significant difference (P<0.001) between concentrations in all the three sub cellular fractions viz. nuclear (Table 8.3.8a), soluble (Table 8.3.8b) and lysosomal (Table 8.3.8c). Between days of exposure a marked significant difference (P<0.001) was observed. Consideration days as well as concentrations (Interaction) a significant difference (P<0.001) was noted in all the three sub cellular fractions. This explains that sub cellular β-glucuronidase activity in the hepatic tissue of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol seemed to be highly significant (P<0.001) in all the three sub cellular fractions.

Table 8.3.9 Multiple Comparison Test (Concentration) (*In- vivo*)

	Groups	Nuclear fraction	Soluble fraction	Lysosomal fraction
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < 0.001.

A subsequent pair wise comparison between various concentrations with respect to control was carried out using Dunnett's method and the results are depicted in Table 8.3.9. This indicated that ethanol at all the three sub lethal concentrations exhibited significant difference (P<0.001) in nuclear, soluble and lysosomal fractions when compared with control.

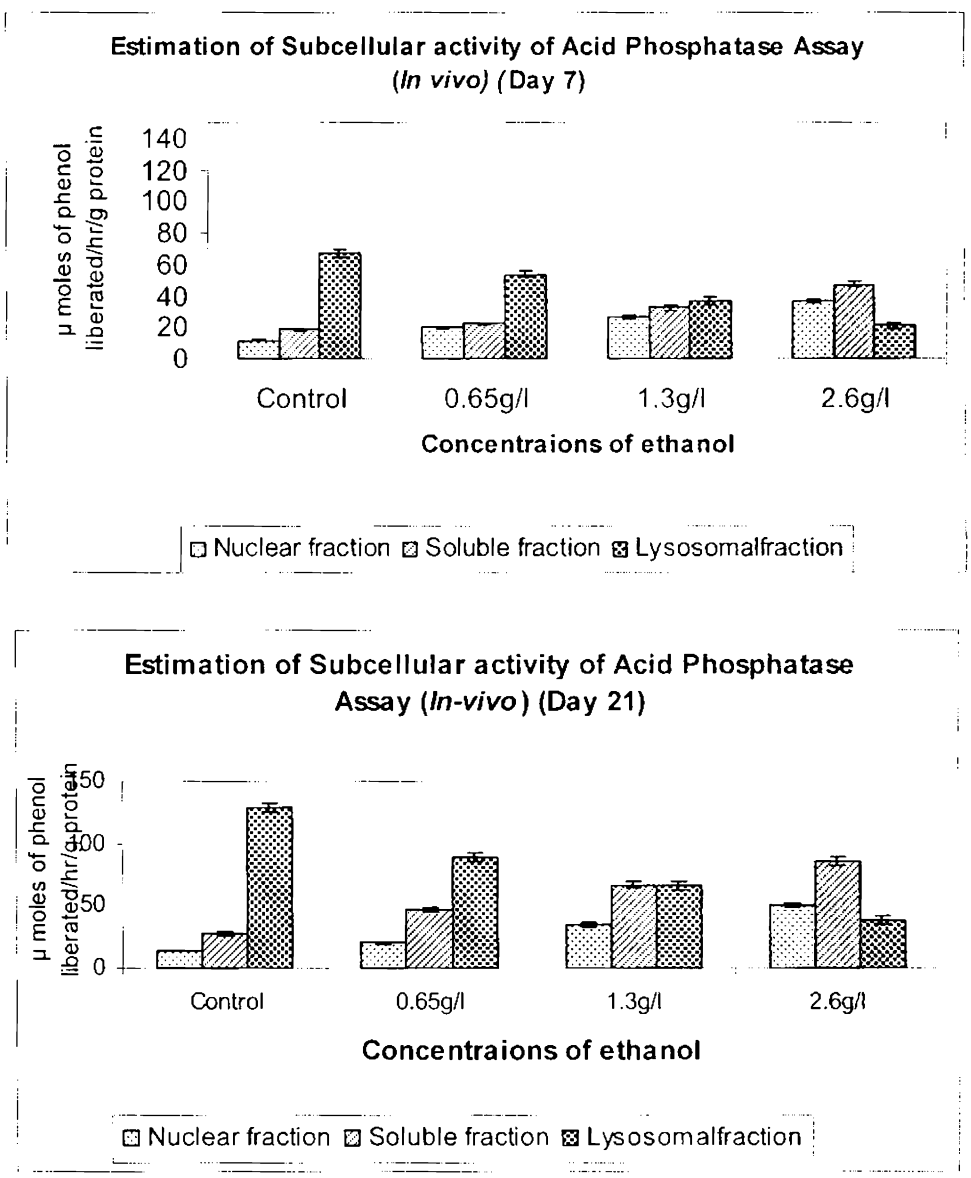
Table 8.3.10 Effect of different concentrations of ethanol on the sub cellular activity of acid phosphatase in *O. mossambicus (In-vivo)*

Sub cellular Fractions		Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Day 7	Nuclear	11.99± 0.9738	20.14± 0.6814	26.69± 1.2613	37.33± 1.3043
	Soluble	18.63± 0.4897	22.32± 0.8055	32.67± 1.7952	47.25± 1.7315
	Lysosomal	67.62± 2.9004	53.95± 2.1870	37.38± 2.6362	21.19± 1.6182
	Ratio Lysosomal to soluble activity	3.633± 0.2038	2.421± 0.1589	1.147± 0.1059	0.449± 0.0453
Day 21	Nuclear	14.02± 0.6788	20.48± 1.1950	34.62± 1.7282	50.37± 1.5792
	Soluble	27.78± 2.3655	47.55± 2.0787	66.57± 2.7574	86.30± 3.5222
	Lysosomal	128.5± 3.4371	88.98± 2.6667	65.83± 3.7034	38.52± 2.9394
	Ratio Lysosomal to soluble activity	4.656± 0.4522	1.875± 0.1152	0.992± 0.0897	0.447± 0.0357

Average of six values in each group ± SD of six observations

The values are expressed as μmoles of phenol liberated/ hour/ gram protein.

Fig 8.3.11 Levels of sub cellular acid phosphatase activity in the hepatic tissue of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol *(In- vivo)*



Acid phosphatase activity was found to be significantly ($P<0.001$) altered in the hepatic tissues of *O. mossambicus* subjected to all the three sub lethal concentrations of ethanol (Figure 8.3.11) with respect to control in both durations of exposure. To validate this ANOVA was carried out and the results obtained are depicted below (Table 8.3.11a to 8.3.11c).

Table 8.3.11a ANOVA table for nuclear acid phosphatase activity *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	6457.395	3	2152.465	1426.249	0.000
Between Days of Exposure	408.773	1	408.773	270.858	0.000
Concentration × Days of Exposure	302.899	3	100.966	66.901	0.000
Error	60.367	40	1.509		
Total	7229.434	47			

df – degrees of freedom

Table 8.3.11b ANOVA table for soluble acid phosphatase activity *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	12772.899	3	4257.633	919.690	0.000
Between Days of Exposure	8639.645	1	8639.645	1866.248	0.000
Concentration × Days of Exposure	1543.012	3	514.337	111.102	0.000
Error	185.177	40	4.629		
Total	23140.733	47			

df – degrees of freedom

Table 8.3.11c ANOVA table for lysosomal acid phosphatase activity *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	30246.316	3	10082.105	1392.289	0.000
Between Days of Exposure	15215.054	1	15215.054	2101.123	0.000
Concentration × Days of Exposure	3041.813	3	1013.938	140.020	0.000
Error	289.656	40	7.241		
Total	48792.839	47			

df – degrees of freedom

Usage of Two factor ANOVA test indicated that acid phosphatase activity exhibited significant difference (P<0.001) between concentrations. After taking into consideration the effect between days of exposure significant difference (P<0.001) was observed. It was understood that considering both days of exposure as well as concentrations (Interaction) significant difference (P<0.001) was obtained in nuclear (Table 8.3.11a), soluble (Table 8.3.11b) and lysosomal (Table 8.3.11c) sub cellular fractions which indicated that acid phosphatase activity was highly significant.

Table 8.3.12 Multiple Comparison Test (Concentration) (*In- vivo*)

Subsequent pair wise comparisons by multiple comparison tests using Dunnett's method is shown below.

	Groups	Nuclear fraction	Soluble fraction	Lysosomal fraction
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]

The values are significant at a=P < 0.001.

Pair wise comparison using Dunnett's method indicated that the the three sub lethal concentrations of ethanol when been compared with control, exhibited significant difference (P<0.001) in all the three sub cellular fractions - nuclear, soluble and lysosomal.

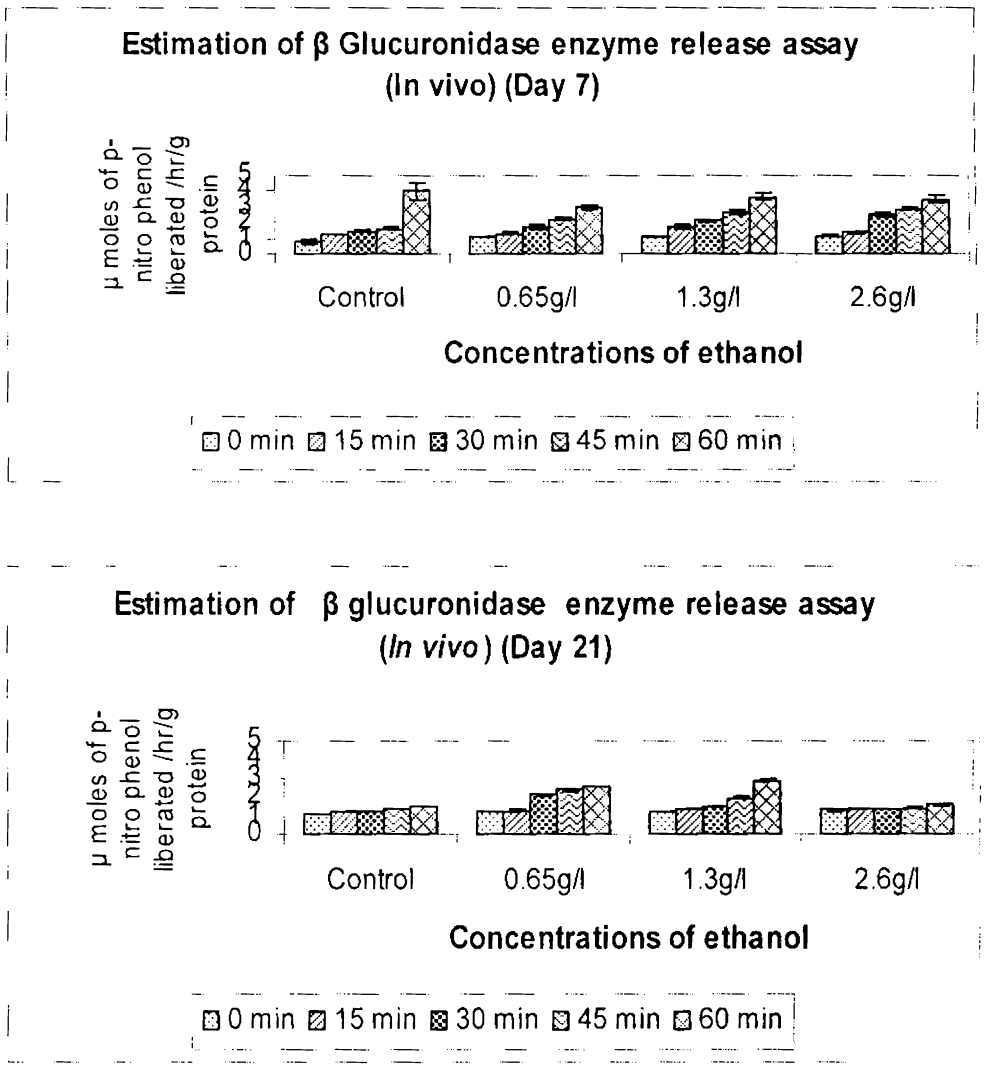
Table 8.3.13 Time dependent release of β-glucuronidase enzyme in *O. mossambicus* exposed for 7 and 21 days *(In- vivo)*

Parameters		Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
Day 7	0 min	0.772± 0.1279	1.034± 0.0156	1.094± 0.0221	1.160± 0.0345
	15 min	1.286± 0.0156	1.311± 0.0784	1.731± 0.1889	1.331± 0.0456
	30 min	1.471± 0.0171	1.650± 0.1544	2.126± 0.0763	2.492± 0.1270
	45 min	1.623± 0.0267	2.247± 0.0524	2.686± 0.1930	2.870± 0.0781
	60 min	3.986± 0.5419	3.010± 0.1911	3.626± 0.2117	3.472± 0.2212
Day 21	0 min	1.118± 0.0165	1.165± 0.0110	1.223± 0.0218	1.295± 0.0135
	15 min	1.175± 0.0105	1.269± 0.0479	1.314± 0.0229	1.359± 0.0220
	30 min	1.248± 0.0203	2.123± 0.0884	1.464± 0.0397	1.377± 0.0097
	45 min	1.348± 0.0251	2.415± 0.0696	1.920± 0.0462	1.416± 0.0090
	60 min	1.507± 0.0321	2.534± 0.0312	2.854± 0.0641	1.557± 0.0735

Average of six values in each group ± SD of six observations

The values are expressed as μ moles of p-nitro phenol liberated /hour/ gram protein.

Fig 8.3.14 Lysosomal enzyme release assay (β–glucuronidase) in the hepatic tissue of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol *(In- vivo)*



From the graph (Figure 8.3.13) it can be understood that the release of β-glucuronidase enzyme from the lysosomal fraction was time dependent and the levels were found to be significant ($P<0.001$). Applications employing ANOVA substantiates the above statement (Table 8.3.13a).

Table 8.3.13a ANOVA Table for β-glucuronidase enzyme release assay *(In- vivo)*

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Time	86.098	4	21.524	114.535	0.000
Between Concentrations	6.487	3	2.162	11.505	0.000
Between Days of Exposure	12.971	1	12.971	69.023	0.000
Error	43.412	231	0.188		
Total	148.968	239			

Three factor ANOVA table illustrated that significant difference (P<0.001) was obtained when time intervals were taken into account. Considering the effect between concentrations as well as between 7 and 21 days of exposure period, significant difference (P<0.001) was found.

Table 8.3.14 Multiple Comparison Test (Time) (*In–vivo*)

Subsequent pair wise comparisons by multiple comparison tests using Tukey is shown below.

	Groups	β-Glucuronidase enzyme release assay
Tukey	0 min Vs 15 min	0.056[d]
	0 min Vs 30 min	0.000[a]
	0 min Vs 45 min	0.000[a]
	0 min Vs 60 min	0.000[a]
	15 min Vs 30 min	0.000[a]
	15 min Vs 45 min	0.000[a]
	15 min Vs 60 min	0.000[a]
	30 min Vs 45 min	0.003[b]
	30 min Vs 60 min	0.000[a]
	45 min Vs 60 min	0.000[a]

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

Pair wise comparison employing Tukey's post hoc test explained all possible interactions of time intervals. From the table indicated above (Table 8.3.14) it is well

understood that when 15 min when compared to 0 minute no significant difference was got. Similarly 45 minute when compared with 30 minute significant difference ($P<0.05$) was obtained. All the other time interval interactions were found to be significant ($P<0.001$).

8.4D Discussion

Exposure to contaminants including organic xenobiotics, results in increased radical generation and the intra lysosomal environment has already been a site of oxyradical production resulting in oxidative damage to membranes, proteins (e.g. carbonyls) and DNA which will undoubtedly contribute to decreased protein synthesis, cell injury and patho-physiological dysfunction. (Livingstone, 2001). Many xenobiotics evoke alteration directly in the bounding membrane of the lysosomes (Moore and Lowe, 1985). A distinct decline in the stability of lysosomal membrane in relation to contaminant burden has been reported by Kohler (1989a, 1990) and Ward (1990). Also, the level of destabilization bears a quantitative relationship to the degree of stress (Bayne *et al.*, 1976, Moore and Stebbing, 1976; Moore *et al.*, 1978). The measurement of lysosomal perturbations in fish liver as integrative biological warning system for biological effect monitoring, was suggested by kohler (1990, 1991). The effect of chemicals on a lysosome membrane can be evaluated by measuring the activity of released enzymes (Dean, 1981). Lysosomal perturbations have been widely used as early indicators of adverse effect to various factors, including pollutant exposure (Galloway *et al.*, 2004; Moore, 2002; Moore and Noble, 2004). In general, lysosome alterations are indicative of early cytopathological responses; they are sensitive but nonspecific to a wide range of contaminants which ultimately affects aquatic ecosystem. The sensitivity of lysosomes to environmental pollutants suggests that lysosomal responses may be considered as early warning systems for detection of the disturbances in the surroundings.

Weeks and Svendsen (1996) stated lysosomal fragility as a promising biomarker in environmental toxicity studies. Alteration in the lysosomal stability is due to the functional modifications in the lysosomes and in certain instances, the

structural changes which are all induced by the stressor and are indicative of cytotoxicity (Moore *et al.*, 1978; Bayne *et al.*, 1978). Investigations at the subcellular level can reveal alteration at an early stage of response, before integrated cellular damage shifts to the level of organ or whole organism. In many instances, the earliest detectable alterations are associated with the lysosomes (Moore, 1985). Lysosomal alterations in fish hepatocytes have been recommended as potential cytological biomarkers for environmental pollutants. Methods used in the determination of lysosomal integrity in fish hepatocytes have been well estabilished (Kohler, 1991; Kohler *et al.*, 1992; Lowe *et al.*, 1992). Lysosomal stability is a good indicator of physiological fitness in fish liver (Allen and Moore, 2004). In the present study a significant decrease ($P<0.001$) in lysosomal fraction of β-glucuronidase and acid phosphatase activities were observed followed by an increase in soluble and nuclear fraction both in *in vitro* studies and *in vivo* studies (Table 8.3.2 to 8.3.12). Alterations to lysosomal structure are usually associated with a reduction in the stability of lysosomal membranes followed by an increase in lysosomal enzyme activities, which may pose a potential risk to the health of the cell or the individual organism (Kohler, 1991; Moore, 1993). Studies conducted by Kohler *et al.* (2002) and Wahli (2002) demonstrated a good dose-response relationship between the severity of lysosomal alterations in liver of European flounder and the levels of xenobiotic pollution. Studies conducted by Camus *et al.* (2000) corroborated that lysosomal membrane of haemocytes of *M. edulis* became more fragile at 0^0 than at 10^0C exhibiting decreased stability of membranes in haemocytes when kept for 1.5 months at 0^0C, supporting the present study. Results of lysosomal enzyme release assay carried out *in vivo* (Table 8.13 and Figure 8.14) revealed significant increase in β-glucuronidase release with time ($P<0.001$) (Table 8.14a). Lysosomal membrane destabilization is a prognostic biomarker for toxicant-induced fish liver dysfunction in biomonitoring programs (Broeg *et al.*, 1999; Kohler *et al.*, 2001, 2002). Ethanol on immediate and prolonged exposure, acted upon lysosomal membranes, causing structural and physiological changes such as lysosomal fragility and release of acid hydrolases. These alterations are components of the inflammatory process that are followed by cell death (Cancio *et al.*, 1995b). Studies conducted on lysosomes in mammals showed increased membrane fragility,

which in turn may lead to cellular destruction and cell death upon heavy metal injury as reported by Sternlieb and Goldfischer (1976). Studies conducted by Tsvetkov *et al.* (1997) showed similar changes in cathepsin D and acid phosphatase which explains lysosome targeted stress. To completely understand toxic responses in the liver and to further apply this knowledge, we need to integrate molecular, biochemical, physiological and morphological findings from the cellular to organ level.

Chapter 9

EFFECT OF ETHANOL ON ANTIOXIDANT PARAMETERS (NON ENZYMATIC AND ENZYMATIC ANTIOXIDANTS) OF *OREOCHROMIS MOSSAMBICUS* (PETERS).

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9.1A Introduction

Pollution of water sources due to xenobiotics plays a major role in the decline of aquatic animals (Henry *et al.*, 2004). The aquatic environment which receives daily substantial amounts of environmental pollutants has the potential to cause oxidative stress in aquatic organisms through free radical and ROS mechanisms. This mainly occurs as a result of the effect of xenobiotics causing the disturbances in the antioxidant enzyme systems and has become an important subject for terrestrial and aquatic toxicology (Ames *et al.*, 1993; Imlay and Linn,

1988; Livingstone, 2001; Stadtman and Berlett, 1997). Fishes used to possess the same biochemical pathways to deal with the toxic effects of endogenous and exogenous agents as mammals (Lackner, 1998). Current knowledge and recent advances of oxidative toxicity by xenobiotics in aquatic organisms provide a fertile field for aquatic toxicology studies (Livingstone, 1998).

Aerobic organisms have developed through evolutionary processes antioxidant defense mechanisms designed to prevent cellular damage from ROS. In general, producing and scavenging of ROS are balanced dynamically (Fang and Zheng, 2002). If excess ROS is produced in metabolism and cannot be scavenged by antioxidant enzymes, it would result in oxygen stress and damage the macromolecules, such as proteins, nucleic acids and lipids. Cells possess a complex defence system to protect themselves from ROS, including non enzymatic scavengers and the main antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Orbea *et al.*, 2002). Aerobic organisms generate superoxide anion radical, ($O_2.^-$) hydrogen peroxide (H_2O_2) and hydroxyl radical (.OH) as a result of oxidative metabolism. Hydroxyl radical (.OH) can initiate lipid peroxidation in tissues (Halliwell and Gutteridge, 1984). The interplay between ROS and antioxidant defenses in living aerobic organisms is connected with a series of intracellular antioxidant enzymes, whose roles are to intercept and inactivate reactive radicals. Also, extracellular low molecular- weight antioxidant molecules (such as ascorbate, uric acid, etc) circulate in biological fluids scavenging free radicals and ROS (Davies, 1995). Free radicals are highly reactive species characterized by one or more unpaired electrons in their outer orbital. Reactive oxygen species (ROS) are constantly generated in cells through processes such as mitochondrial electron transport, metabolism of xenobiotics, and inflammation (Cnubben *et al.*, 2001). In the course of evolution, aerobic organisms have evolved a suite of enzymatic and non-enzymatic proteins to prevent damage of lipid, protein and DNA caused by endogenous free radicals produced during their metabolism (Ahmad, 1995; Di Giulio, 1991).

The antioxidant defense system of living organisms are subdivided into enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), and

glutathione peroxidase (GPX). The non enzymatic antioxidant systems are mainly substances of low molecular weight, such as vitamins C and E, urate, retinyl esters, β-carotene, glutathione (GSH), etc. (Kohen and Nyska, 2002). Endogenous and exogenous oxidative challenges have endowed living cells of aerobic organisms with sophisticated antioxidant systems (enzymatic and non enzymatic) to regulate oxidative stress (Chow, 1988). Various studies with experimental animals demonstrated that free radical peroxidation by xenobiotics increases MDA in tissue samples (Di Pierro *et al.*, 1992). Fish and other aquatic organisms exhibits a variety of changes in enzymatic and low molecular-weight antioxidant defenses after exposure to various pollutants with oxidative potential (Regoli *et al.*, 2002a,b; Winston and Di Giulio, 1991). Several studies in aquatic organisms demonstrated the importance of enzymatic antioxidant defenses in protecting cellular systems from oxidative stress induced by xenobiotics. Recent investigations of changes in antioxidant defenses showed that they can be used as biomarkers of oxidative stress by various pollutants in aquatic organisms. The main damage induced by ROS results in alterations of cellular macromolecules such as membrane lipids (lipid peroxidation), DNA, and/or proteins. Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radical, are generated from normal metabolic processes in all oxygen-utilizing organisms. The damage from ROS includes lipid peroxidation, cross-linking and inactivation of proteins, DNA and RNA breaks, and cell death (Halliwell and Gutteridge, 1984; Hu *et al.*, 1995; Wiseman and Halliwell, 1996). To prevent damage from oxidative stress, cells maintain these ROS at a steady-state level by a variety of enzymatic and non enzymatic antioxidant systems. Estimation of lipid peroxidation (LPO) in particular has been found to have high predictive importance as revealed from a credible number of research papers describing its use as a biomarker of effect (Lackner, 1998). In addition to LPO, other parameters of both antioxidant enzymes and non-enzymatic antioxidants have also been successfully employed in aquatic biomonitoring studies (Hasspieler *et al.*, 1994; Filho, 1996). Lipid peroxidation has been used successfully as a measure of xenobiotic-induced oxidative stress. Lipid peroxidation has received increasing attention as a mechanism of toxicity for a variety of organic and inorganic environmental pollutants (Gutteridge and Halliwell,

1990). Glutathione depletion promotes lipid peroxidation as well. Like all aerobic organisms, fishes are susceptible to the attack of reactive oxygen species and have developed antioxidant defenses demonstrated by research primarily dating to the 1980s. Specially adapted enzymes, such as catalase (CAT), superoxide dismutase (SOD), and enzymes dependent on glutathione (glutathione peroxidase, GPX, and glutathione reductase, GR) have been detected in most fish species investigated to date (Rudneva, 1997). Together with these enzymes, lower-molecular- weight antioxidants, such as carotenoids, vitamins E, K and C, amino acids and peptides (glutathione), have been detected in antioxidant defenses in fish. High levels of non-enzymatic antioxidants have been detected in marine invertebrates and fish (Kossmann, 1988; Mezes, 1986).

Ethanol-induced oxidative stress is linked to the metabolism of ethanol which involves the following enzymes such as alcohol dehydrogenase, microsomal ethanol oxidation system (MEOS) and catalase. Each metabolic pathway of ethanol produces specific metabolic and toxic disturbances. It has been suggested that ethanol may cause tissue damage through lipid peroxidation (Montoliu *et al.*, 1994). The metabolic effects of alcohol are due both to its direct action and to that of its first metabolite acetaldehyde, and can also be connected with the changes in redox state. Ethanol metabolism generates reactive oxygen species by inducing lipid peroxidation and decreases cellular levels of antioxidant enzymes.

In living cells, there is a continuous production of free radicals and many pathologies are believed to result from damage to tissue initiated by them due to the production of the radicals overwhelming the defense mechanisms. The antioxidant enzymes and other antioxidants provide the cells with protection against oxidative stress. GST in some species of fish has been used as a biomarker for aquatic biomonitoring (Van der Oost *et al.*, 2003). GSH is an endogenous compound that protects the fish against xenobiotics. Biomarkers measured at the molecular or cellular level in fish have been proposed as sensitive "early warning" tools for biological effect measurements in environmental quality assessments (Van der Oost *et al.*, 2003). Indeed, the induction of these antioxidant responses and LPO have been commonly employed as biomarkers of oxidative stress (Ahmad *et al.*, 2000;

Di Giulio, 1991; Livingstone, 1993; Lackner, 1998). Hence the present study was focused to investigate the effect of ethanol on enzymatic and non enzymatic antioxidant enzymes which also can serve as biomarkers of ethanol induced oxidative stress.

9.2B Materials and Methods

Collection, maintenance of fish, acclimatization of fish and determination of LC_{50}, was the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

9.2B.1 Methods used for the biochemical analysis

The following are the parameters studied under lipid peroxidation.

a. Estimation of Lipid peroxidation

The product of lipid peroxidation, malondialdehyde has been identified and was estimated by the method of Niehaus and Samuelson (1968). In this method, malondialdehyde was measured by their reactivity with thiobarbituric acid (TBA) in acidic condition to generate a pink coloured chromophore, which was read at 535nm.

Reagents

TCA-TBA-HCl reagent: 15% w/v Trichloroacetc acid, 0.375% w/v thiobarbituric acid (TBA) in 0.25N HCl, 0.1M Tris HCl buffer (pH 7.5).

Procedure

Homogenate of gill, muscle, liver, heart and kidney tissues were prepared in Tris HCl buffer of pH 7.5. 1.0 ml of the tissue homogenate was treated with equal amount of TCA-TBA-HCl reagent. The contents were mixed thoroughly and was t heated for 15 minutes in a boiling water bath. After cooling, the tubes were centrifuged for 10 minutes at 600g.The supernatant was taken. The absorbance of the sample was read spectrophotometrically at 535 nm against a reagent blank that contained no tissue extract. The extinction coefficient for malondialdehyde is

$1.56\times10\text{-}5/M^{-1}/cm^{-1}$.The values were expressed as millimoles/100 g wet weight of the tissue.

b. Estimation of superoxide dismutase (SOD; E.C 1.15.1.1)

Principle

Superoxide dismutase activity was estimated by the method of Das *et al.* (2000).

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with naphthylethylenediamine to produce a red azo compound whose absorbance is measured at 543 nm.

Reagents

1. 50 mM Phosphate buffer, pH 7.4
2. 20 mM L-Methionine
3. 1% (v/v) Triton X-100
4. 10 mM Hydroxylamine hydrochloride
5. 50 µM EDTA
6. 50 µM Riboflavin
7. Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride.

Procedure

The tissue homogenate of gill, muscle, liver, heart and kidney tissues were prepared in Tris HCl buffer of pH 7.5. Pipetted 1.5 ml aliquot of the reaction mixture in a test tube which consists of 0.3 ml each of phosphate buffer, methionine, triton x-100, hydroxylamine hydrochloride and EDTA. To this 100 µl of the sample was added followed by pre incubation at 37^0C for 5 min. Into this 80 µl of riboflavin was added and the tubes were exposed for 10 min to 200W Philips

fluorescent lamp. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time (10 minutes), 1.0 ml of Griess reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm against buffer taken as blank. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

c. Estimation of Catalase (CAT; E.C 1.11.1.6)

Principle

Catalase activity was estimated according to the method of Sinha (1972).

Catalase causes rapid decomposition of hydrogen peroxide to water.

H_2O_2 ----------→ $H_2O + O_2$

The method is based on the fact that dichromate in acetic acid reduces to chromic acetate when heated in the presence of H_2O_2 with the formation of perchloric acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically at 610 nm. Since dichromate has no absorbancy in this region, the presence of the compound in the assay mixture do not interfere with the colorimetric determination of chromic acetate. The catalase preparation is allowed to split H_2O_2 for different periods of time. The reaction is stopped at specific time intervals by the addition of dichromate/acetic acid mixture and the remaining H_2O_2 is determined by measuring chromic acetate colorimetrically after heating the reaction.

Reagents

1. 0.01 M Phosphate buffer, pH 7.0
2. 0.2 M Hydrogen peroxide
3. Stock dichromate/acetic acid solution: Mixed 5% potassium dichromate with glacial acetic acid (1:3 by volume).
4. Working dichromate/acetic acid solution: The stock was diluted to 1:5 with distilled water to make the working dichromate/acetic acid solution.

Procedure

Weighed sample of gill, muscle, liver, heart and kidney tissues were prepared in 0.01M PO_4 buffer of pH 7.0. The assay mixture contained 0.5 ml of H_2O_2, 1.0 ml of PO_4 buffer and 0.4 ml of distilled water. 0.2 ml of the enzyme was added to initiate the reaction. 2.0 ml of the dichromate/acetic acid reagent was added after 0, 30, 60, 90 seconds of incubation. To the control tube, enzyme was added after the addition of the acid reagent. The tubes were then heated for 10 minutes and the colour developed was read at 610 nm against distilled water taken as blank. The activity of catalase was expressed as µmole of H_2O_2 decomposed/min/mg protein.

d. Estimation of glutathione peroxidase (GPx; E.C 1.11.1.9)

Principle

Glutathione peroxidase activity was estimated according to the method of Rotruck *et al.* (1973). A known amount of enzyme preparation was allowed to react with hydrogen peroxide in the presence of GSH for a specified time period. Then the remaining GSH was measured by the method of Ellman.

$$2GSH+H_2O_2 \xrightarrow{\text{Se-GPx}} GSSG+2H_2O$$

Reagents

1. 0.4 M Sodium phosphate buffer, pH 7.0
2. 10 mM Sodium azide
3. 2.5 mM Hydrogen peroxide
4. 4 mM Reduced glutathione
5. 10% TCA
6. 0.3 M Phosphate ($Na_2 HPO_4$) solution
7. 0.04% DTNB in 1% sodium citrate
8. Reduced glutathione standard: 20 mg reduced glutathione was dissolved in 100 ml of water.

Procedure

Homogenate of gill, muscle, liver, heart and kidney tissues prepared in Tris-HCl buffer of pH 7.5 were taken for the assay. To 0.4 ml of buffer, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, 0.1 ml of H_2O_2, 0.2 ml of enzyme and 1.0 ml of dist. H_2O water were added and made up to a final incubation volume of 2.0 ml. The tubes were incubated for 0, 30, 60 and 90 seconds respectively. The reaction was then terminated by the addition of 0.5 ml of TCA. To determine the glutathione content, 2.0 ml of the supernatant was removed by centrifugation and to this 3.0 ml disodium hydrogen phosphate solution and 1.0 ml of DNTB reagent was added. The colour developed was read at 412 nm against buffer taken as blank. Standards in the range of 200-1000 µg were taken and treated in the similar manner. The activity was expressed in terms of µg of glutathione utilized/min/mg protein.

e. Estimation of glutathione-S-transferase (GST; E.C 2.5.1.18)

Principle

Glutathione-S- transferase activity was estimated according to the method of Habig *et al.* (1973). Glutathione-S-transferase catalyses the reaction of 1-chloro 2.4 dinitrobenzene (CDNB) with the sulphhydryl group of glutathione.

$$\text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB-S-glutathione}$$

The conjugate, CDNB-glutathione, absorbs light at 340 nm and the activity of the enzyme can therefore be estimated by measuring the increase in absorbance.

Reagents

1. 0.5 M phosphate buffer, pH 6.5
2. 30 mM CDNB in 95% ethanol (30 mg of CDNB was dissolved in 5 ml of distilled H_2O)
3. 30 mM reduced glutathione (14 mg of reduced glutathione was dissolved in 1.5 ml of distilled H_2O)

Procedure

The tissue homogenate of gill, muscle, liver, heart and kidney tissues prepared in Tris HCl buffer of pH 7.5 was taken for the assay. To 1.0 ml of buffer, 0.1 ml of sample, 1.7 ml of water and 0.1 ml of CDNB were added .The tubes were then incubated at 37^0C for 5 minutes. After suitable period of incubation, 0.1 ml of reduced glutathione was added. The increase in optical density of the enzyme was measured against that of the blank at 340 nm. The enzyme activity is calculated in terms of µmoles of CDNB conjugate formed/min/mg protein.

f. Estimation of glutathione reductase (GR; E.C 1.6.4.2)

Principle

Glutathione reductase activity was estimated according to the method of Beutler (1984). Glutathione reductase catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) and is assayed by measuring the decrease in absorbance at 340 nm.

$$NADPH\ (NADH) + H^+ + GSSG \xrightarrow{GR} NADP^+(NAD)^+ + 2GSH$$

Reagents

1. 0.3 M phosphate buffer, pH 6.8
2. 25 mM EDTA (93 mg of EDTA was dissolved in 10ml of distilled H_2O)
3. 12.5 mM oxidized glutathione (11.5 mg of oxidized glutathione was dissolved in 1.5 ml of distilled H_2O)
4. 3 mM NADPH (2.5 mg of NADPH was dissolved in 1 ml of distilled H_2O)

Procedure

The tissue homogenate of gill, muscle, liver, heart and kidney tissues were prepared in 0.1M Tris HCl buffer of pH 7.5 and was taken for the assay.

To 0.2 ml of tissue sample, 1.5 ml of buffer, 0.5 ml EDTA, 0.2 ml GSSG and0.1 ml of NADPH was added. The decrease in optical density of the enzyme was measured against that of the blank at 340 nm. The enzyme activity is calculated in terms of μmoles of NADPH oxidized/min/mg protein.

g. Estimation of Conjugated dienes (CD)

The concentration of conjugated dienes was estimated according to the method of Retnagal and Goshal (1966).

Reagents

1. Tris HCL buffer : 0.1 M pH 7.5
2. Chloroform : methanol : 2:1 (v/v)
3. Cyclohexane AR

Procedure

Weighed sample of gill, muscle, liver, heart and kidney tissues were homogenized in a known volume of the 0.1M Tris HCl buffer of pH 7.5. An aliquot of the homogenate was shaken with chloroform methanol (2:1) and the lower layer was recovered. It was then evaporated to dryness. The contents present in the tube was redissolved in 1.5 ml of known volume of cyclohexane. The absorbance was then read at 233nm against cyclohexane reagent taken as blank. The amount of conjugated dienes obtained was expressed in terms of millimoles/100g tissue. Molar extinction coefficient of conjugated dienes was $2.52 \times 10^{4} M^{-1} cm^{-1}$.

h. Estimation of Hydroperoxides

Hydroperoxides was estimated by the method of Mair and Hall (1977).

Reagents

1. Potassium iodide (KI)
2. 0.5% Cadmium acetate

Procedure

1ml of the tissue homogenate of the different tissues such as gill, muscle, liver, heart and kidney homogenized in 0.1M Tris HCl of pH7.5 was taken for the assay. The homogenate was mixed thoroughly with 5 ml of chloroform: methanol (2:1) followed by centrifugation at 1000g for 5 minutes to separate the phases. 3 ml of the lower chloroform layer was recovered using a syringe and was placed in a test tube. It was then dried in a 45^0C water bath under a stream of nitrogen. 1 ml of acetic acid: chloroform (3:2) mixture followed by 0.05 ml of KI was quickly added and the test tubes were stoppered and mixed. The tubes were placed in the dark at room temperature for exactly 5 minutes followed by the addition of 3 ml of cadmium acetate. The solution was mixed and was centrifuged at 1000g for 10 minutes. The absorbance of the upper phase was read at 353 nm against a blank containing the complete assay mixture except the tissue homogenate. Molar extinction coefficient of hydroperoxide was 1.73×10^4 M^{-1} cm^{-1}.

i. Estimation of total reduced glutathione

Principle

Total reduced glutathione was estimated according to the method of Moron *et al.* (1979). Glutathione (GSH) was measured by its reaction with DTNB to give a compound that absorbs at 412 nm.

Reagents

1. Metaphosphoric acid: Dissolved 1.67 g of glacial metaphosphoric acid, 0.2g of EDTA and 30 g of NaCl in 100 ml of distilled water.
2. 0.4 M Na_2HPO_4
3. DTNB reagent: 40 mg DTNB was dissolved in 100 ml of 1% trisodium citrate.
4. Standard glutathione: 20 mg reduced glutathione was dissolved in 100 ml of distilled water.

Procedure

The tissue homogenate of gill, muscle, liver, heart and kidney tissues prepared in 0.1M Tris HCl buffer of pH 7.5 was taken for the assay. To 1.0 ml of tissue homogenate, 4.0 ml of metaphosphoric acid was mixed. The precipitate obtained was removed by centrifugation. To 2.0 ml of the supernatant, 2.0 ml disodium hydrogen phosphate and 1.0 ml of DTNB reagent was added. The absorbance was read within 2 minutes at 412 nm against a reagent blank. A set of standards were also treated in the above manner. The amount of glutathione was expressed as µg of glutathione obtained/mg protein.

j. Estimation Vitamin A

Principle

Vitamin A content present in the tissues were estimated by Nield *et al.* (1963). The method is based on the measurement of the interaction of vitamin A with trifluroacetic acid, the intensity of which is a function of the concentration of vitamin A which is measured at 620 nm. A correction for the absorbance contribution by carotene is necessary.

Reagents

1. 2 N KOH
2. 90% alcohol
3. Petroleum ether
4. Trifluro acetic acid
5. Chloroform TFA reagent: Mixed 1.0 ml of TFA and 2.0 ml of chloroform. This was prepared freshly before use.
6. Vitamin A stock standard (160 µg/ml): Transferred 16 mg of all trans retinyl acetate to 100 ml standard flask and was made upto 100 ml with anhydrous chloroform.

7. Vitamin A working solution: Pipetted out 0.2-1.0 ml of stock vitamin A solution and was made up to 100 ml with anhydrous chloroform with corresponding concentration of 3-15µg respectively.
8. β-carotene stock standard (200 µg/ml) : Transferred 20 mg of β-carotene to 100 ml standard flask. Dissolved in approximately 4 ml of chloroform and was diluted to 100 ml with petroleum ether.
9. Carotene working standard: Pipetted out 0.05 – 0.2 ml of β-carotene stock and made up to 1.0 ml with petroleum ether. It will have a concentration corresponding to 1-4 µg respectively.

Procedure

To 1.0 ml of tissue homogenate of gill, muscle, liver, heart and kidney tissues prepared in 0.1M Tris HCl buffer of pH 7.5, 1.0 ml of saponification mixture (2 N KOH in 90% alcohol) was added and heated under gentle reflux for 20 minutes at 60^0C. 25 ml of water was added to the mixture after cooling it to room temperature and the solution was then transferred to a separating funnel. It was then extracted thrice with using 25, 15 and 10 ml of petroleum ether (40– 60^0C). The extracts were pooled and were washed with 50 – 100 ml of distilled water repeatedly until the wash water was free of alkali. The petroleum ether extract obtained was then dried by adding anhydrous sodium sulphate. The volume of the extract was noted. 3.0 ml of petroleum ether phase was transferred to a cuvette and was read at 420 nm against petroleum ether taken as blank without delay to prevent evaporation of the solvent and destruction of carotenoids by light. This reading was marked as A_1. The β- carotene working standards were measured at 450 nm. The aliquots were evaporated to dryness at 60^0C in a water bath. The residue was taken immediately and 2.0 ml TFA reagent were added to it. The mixture was rapidly transferred to a cuvette and the absorbance was measured at 620 nm exactly after the addition of TFA reagent. This reading was marked as A_2. The vitamin A working standard obtained was read at 620 nm.

Calculation

For accurate calculation of the vitamin A content, it was necessary to correct for the absorbance contributed by carotene at 620 nm.

$A_3=A_2-A_1$

A_1=Absorbance of carotene at 450 nm

A_2=Absorbance at 620 nm due to both carotene and vitamin A.

A_3=Absorbance at 620 nm of vitamin A.

$$\text{Sample} = \frac{A_3 \times \mu g \text{ retinol calibarator/cuvette} \times 3 \times \text{total volume}}{A_{620} \text{ retinol calibarator} \times 2 \times \text{gram}}$$

3= volume of petroleum ether from 1.0 ml extract

2= Aliquot of the petroleum ether used for the assay

1= Tissue extract taken from initial sample

The results were expressed as µg/g tissue.

k. Estimation of ascorbic acid (Vitamin C)

Principle

Vitamin C content present in the tissues were estimated by Omaye *et al.* (1979). Ascorbic acid is oxidized by copper (Cu^{2+}) to form dehydroascorbic acid and diketoglutaric acid. These products are treated with 2, 4 dinitrophenyl hydrazine to form the derivative of bis 2, 4 dinitro phenyl hydrazine. This compound in strong sulphuric acid undergoes a rearrangement to form a product with an absorption band that is measured at 520 nm. The reaction is run in the presence of thiourea to provide a mildly reducing medium which helps to prevent interference from non-ascorbic acid chromogens.

Reagents

1. 5% TCA
2. 65% H_2SO_4
3. DTCS reagent: 3 g of 2, 4 dinitrophenyl hydrazine, 0.4 g thiourea and 0.05 copper sulphate were dissolved in 9 N sulphuric acid and was made up to 100 ml with the same.

4. Standard ascorbic acid solution: Standard in the range of 4 – 20 µg/ml were prepared in 5% oxalic acid.

Procedure

One ml of tissue homogenate of gill, muscle, liver, heart and kidney tissues prepared in 0.1M Tris HCl buffer of pH 7.5 was taken for the assay. The homogenate was precipitated with 5% ice-cold TCA. It was centrifuged for 20 minutes at 3,500 g. 1.0 ml of the supernatant obtained was mixed with 0.2 ml of DTCS reagent and was incubated for 3 hours at 37^0C. Then 1.3 ml of ice-cold 65% sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for an additional 30 minutes. Absorbance was determined against distilled water taken as blank at 520 nm. The results obtained were expressed as µg/mg protein.

l. Estimation of Vitamin E (α-tocopherol)

Principle

Vitamin E content present in the tissues were estimated by Varley (1976). α-tocopherols can be estimated using emmerie-engel reaction that involves the reduction of ferric ions to ferrous ions by α -tocopherols, which then forms a red coloured complex with 2,2' dipyridyl. Tocopherols and carotenes are first extracted with xylene and the extinction read at 460 nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520 nm.

Reagents

1. Absolute ethanol
2. Xylyene
3. 2,2 dipyridyl : Dissolved 1.3 g of 2,2' dipyridyl in one litre of n-propanol
4. Ferric chloride: Dissolved 1.2 g of $FeCl_3.6H_2O$ or 720 mg of anhydrous ferric chloride in one litre of ethanol.
5. Standard D L-α-tocopherol: Dissolved 10 mg/L in absolute ethanol. 91 mg of α-tocopherol is equivalent to 100 mg of tocopherol acetate.

6. Sample extraction: Weighed 0.1 g of the tissues and were homogenized in a blender. It was then transferred to a conical flask. To this added 5 ml of 0.1 N sulphuric acid slowly without shaking. All the tubes were stoppered and were allowed to stand overnight. The next day, the contents of the flask were shaken vigorously and was filtered through Whatmann No.1 filter paper, by discarding the initial 1-2 ml of the filtrate. Aliquots of the filtrate was used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes named as test, standard and blank, pipetted out 1.5 ml of each tissue extract, 1.5 ml of the standard and 1.5 ml of water respectively. To the test and blank added 1.5 ml ethanol and to the standard added 1.5 ml of water. Added 1.5 ml of ethanol to test and blank and to the standard added 1.5 ml of water. Added 1.5 ml of xylene to all the tubes. The tubes were stoppered, mixed well and was centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube, taking care not to include any ethanol or protein. Added 1.0 ml of 2.2 dipyridyl reagent to each tube. The tubes were stoppered and was mixed well. Pipetted out 1.5 ml of the mixtures into spectrophotometer cuvettes and read the absorbance of test and standard against the blank at 460 nm. Then in turn beginning with the blank, added 0.33 ml of ferric chloride solution. Mixed well and after exactly 1.5 minutes read test and standard against the blank at 520 nm. The amount of vitamin E can be calculated using the formula

$$\text{Vitamin E } (\mu g/g) = \frac{(\Delta A_{520nm} - \Delta A_{450}nm \times \text{conc[S]} \times 0.29 \times \text{Total volume}}{\Delta A_{520\,nm} \times \text{Vol taken for experiment} \times \text{weight of the sample}}$$

m. Estimation of total sulphhydryl group

Principle

Total sulphydryl groups were estimated according to the method of Sedlack and Lindsay (1968). The sulphhydryl groups present in tissues are determined using the Ellman's reagent. In this method DTNB was reduced by sulphydryl group (SH) to form 1 mole of 2-nitro 5- mercaptobenzoic acid per mole of SH.

Reagents

1. 0.01 M DTNB in absolute methanol
2. 0.2 M Tris HCl buffer, pH 8 containing 0.02 M EDTA
3. 0.02. M EDTA

Procedure

A known amount of tissue were homogenized in 8.0 ml of 0.02 M EDTA. 0.2 ml of tissue homogenate was mixed in 15 ml test tubes with 1.5 ml buffer and 0.1 ml of DTNB. The mixture was made up to 10 ml with absolute methanol. A reagent blank without the sample were prepared in the same manner. The test tubes were stoppered and allowed to stand for 15 min with occasional shaking. The reaction mixture was centrifuged at 3,000 g for 15 min at room temperature. The absorbance of the clear supernatant was read at 420 nm. Calibration curves were obtained with reduced glutathione as standard. The values were expressed as μGSH/mg protein.

n. Estimation of peroxidase

Principle

Peroxidase activity in tissues were estimated by the method of Addy and Goodman (1972).

Pyrogallol is used as a substrate for the assay of peroxidase

$$\text{Pyrogallol} + H_2O_2 \xrightarrow{\text{POD}} \text{oxidised pyrogallol} + 2H_2O$$

The rate of the formation of oxidized pyrogallol is a measure of peroxidase activity and was measured at 420 nm.

Reagents

1. 0.1 M Phosphate buffer, pH 6.5
2. 0.05 M pyrogallol: Dissolved 630 mg of pyrogallol in 100 ml of 0.1 M phosphate buffer, pH 6.5.
3. 1% Hydrogen peroxide

Procedure

The tissue homogenate of gill, muscle, liver, heart and kidney prepared in 0.1M Tris HCl buffer of pH 7.5 was employed for the assay. Pipetted out 3.0 ml of 0.05 M pyrogallol solution and 0.1 ml of enzyme extract into a test tube. Adjusted the spectrophotometer to read '0' at 430 nm. Added 0.5 ml of 1% H_2O_2 into the test cuvette. Mixed and recorded the change in absorbance for every 30 seconds upto 3 min. The difference in OD change per minute with and without enzyme addition was a measure of peroxidase acitivity. The activity is expressed in terms of nano moles of pyrogallol/min/mg protein.

o. Estimation of glucose - 6 - phosphate dehydrogenase (E.C 1.1.1.49)

Principle

Glucose-6-phosphate dehydrogenase activity was estimated according to the method of Balinsky and Bernstein (1963). Glucose 6 phosphate dehydroenase was assayed by measuring the increase in absorbance which occurs at 340 nm when NADP was reduced to NADPH. This reaction takes place when electrons were transferred from glucose 6 phosphate to NADP in the reaction catalysed by glucose-6- phosphate dehydrogenase.

Reagents

1. 0.1 M Tris HCl buffer, pH 8.2

A: 0.1 M solution of Tris (12.1 g of Tris was dissolved in 1000 ml of distilled water)

B: 0.1M HCl

Mixed 50 ml of solution A and 21.9 ml of B and diluted to a total of 200 ml.

2. 0.2 mM NADP
3. 0.1 M Magnesium chloride
4. 6 mM glucose-6-phosphate

Procedure

Homogenate of gill, muscle, liver, heart and kidney tissues were prepared in Tris HCl buffer of pH 7.5. To 0.1 ml of Tris HCl buffer, 0.2 ml of NADP, 0.2 ml of magnesium chloride, 1.0 ml water and 0.2 ml of enzyme were taken in a cuvette. The reaction was started by the addition of 0.2 ml of glucose 6 phosphate and the increase in OD was measured at 340 nm for 3 minutes. The activity is expressed in terms of units/mg protein, in which one unit is equal to the amount of enzyme that brought about a change in OD of 0.01 /min

Estimation of tissue total protein

Total protein content in tissues were estimated by the method of Lowry *et al.* (1951) as described in chapter 5,Section5.1B Experiment no: h.

The CO-NH group (peptide bond) present in the protein molecule reacts with copper sulphate in alkaline medium to give a blue colour, which was read at 620nm.

9.3C RESULTS

Effect of different sub lethal concentrations of ethanol exposed for 7 and 21 days on tissues such as gill, muscle, liver, heart and kidney of *O. mossambicus* were given in tables 9.3.1 to 9.3.32 and in figures 9.3.2 to 9.3.30. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), peroxidase (POD), glucose-6-phosphate dehydrogenase (G-6-PD), detoxifying enzymes such as glutathione reductase (GR), glutathione-S-transferase (GST), total reduced glutathione as well as lipid peroxidation products such as conjugated dienes (CD), hydroperoxides (HP) and malondialdehyde (MDA) and non enzymatic antioxidants such as vitamin A, vitamin C, vitamin E, total sulphydryl groups (TSH) were studied. Statistical analysis for all biochemical parameters was carried out using Three Factor ANOVA assuming homogeneity of variance. Where differences were significant, multiple comparisons were carried out by Dunnett's method followed by Tukey's test.

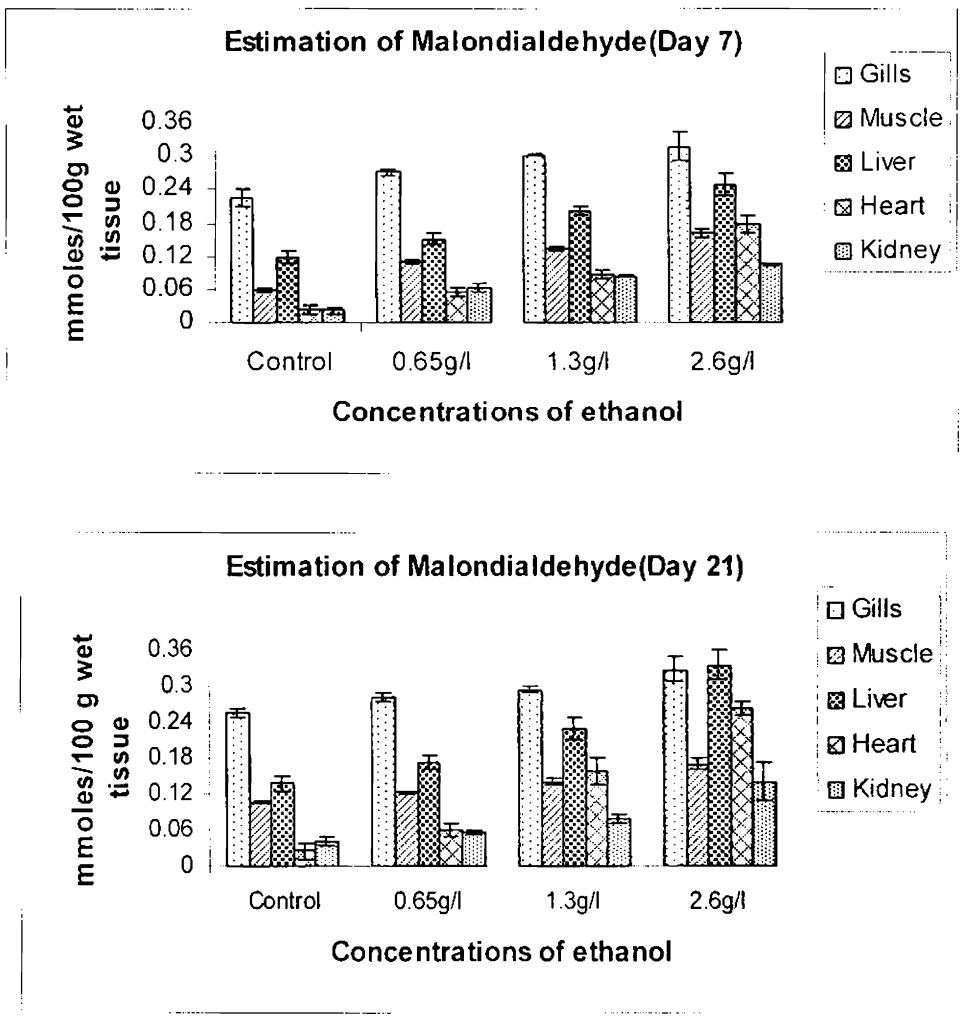
Table 9.3.1 Effect of sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 days and 21 days on malondialdehyde levels.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	0.224± 0.0157	0.271± 0.0076	0.303± 0.0039	0.318± 0.0239
	Muscle	0.059± 0.0048	0.112± 0.0031	0.135± 0.0048	0.162± 0.0088
	Liver	0.119± 0.0108	0.152± 0.0087	0.203± 0.0085	0.249± 0.0183
	Heart	0.024± 0.0061	0.055± 0.0079	0.087± 0.0067	0.179± 0.0167
	Kidney	0.022± 0.0054	0.062± 0.0071	0.085± 0.0032	0.106± 0.0020
21 days of Exposure	Gills	0.257± 0.0074	0.282± 0.0080	0.293± 0.0059	0.328± 0.0210
	Muscle	0.107± 0.0035	0.122± 0.0025	0.140± 0.0053	0.171± 0.0109
	Liver	0.137± 0.0120	0.173± 0.0125	0.228± 0.0193	0.335± 0.0249
	Heart	0.025± 0.0121	0.061± 0.0107	0.157± 0.0221	0.262± 0.0106
	Kidney	0. 040± 0.0069	0.056± 0.0037	0.080± 0.0073	0.141± 0.0306

Values are expressed as milli moles/100 g wet weight of the tissue.

Average of six values in each group ± SD of six observations.

Figure 9.3.2 Levels of malondialdehyde in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different sub lethal concentrations of ethanol.



A significant increase ($P<0.001$) in malondialdehyde level was noted in the tissues (Viz. gills, muscle, liver, heart and kidney) of *O. mossambicus* exposed to the three sub lethal concentrations of ethanol as compared to the control group (Figure 9.3.2). Investigations using ANOVA substantiates the above statement and the results are shown below (Table 9.3.2a).

Table 9.3.2a Three – Factor ANOVA table for malondialdehyde

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	1.367	4	0.342	477.189	0.000
Between Concentrations	0.505	3	0.168	235.284	0.000
Between Days of Exposure	0.033	1	0.033	45.649	0.000
Error	0.165	231	0.001		
Total	2.070	239			

df- degrees of freedom

Three Factor ANOVA table revealed that malondialdehyde levels varied significantly between tissues ($P<0.001$). Between concentrations there was a significant difference ($P<0.001$). Also while taking into consideration the effect between 7 and 21 days significant difference ($P<0.001$) was observed.

Table 9.3.3 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 days and 21 days on the level of total reduced glutathione.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol 0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	11.03± 0.4202	8.627± 0.4958	7.227± 0.6251	4.955± 0.5709
	Muscle	13.580± 1.8342	4.787± 0.6760	3.805± 0.0675	2.200± 0.2768
	Liver	2.420± 0.2490	2.131± 0.0425	1.547± 0.1315	0.950± 0.0318
	Heart	1.446± 0.0255	0.847± 0.0271	0.712± 0.0320	716± 0.0486
	Kidney	1.411± 0.0303	1.173± 0.0134	0.912± 0.0506	0.708± 0.0324
21 days of Exposure	Gills	11.06± 0.5288	8.655± 1.3134	5.504± 0.8313	3.935± 0.3655
	Muscle	6.326± 0.6039	5.145± 0.5507	2.791± 0.3270	1.679± 0.1742
	Liver	1.583± 0.0593	1.209± 0.0499	0.933± 0.1052	0.710± 0.0793
	Heart	1.273± 0.3476	1.078± 0.0316	0.261± 0.0064	0.183± 0.0094
	Kidney	0. 977± 0.0240	0.906± 0.0335	0.682± 0.0788	0.413± 0.0347

Values are expressed as μg GSH/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.4 Levels of total reduced glutathione in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.

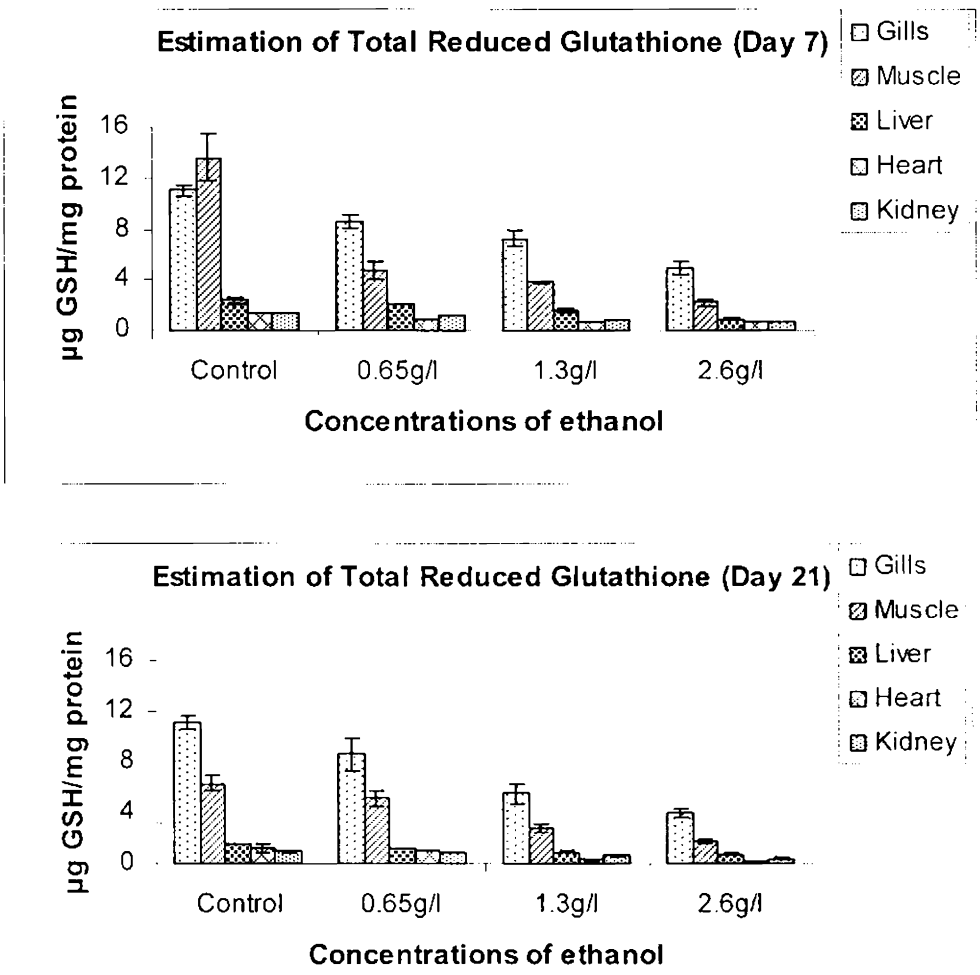


Figure 9.3.4 clearly indicates significant decrease ($P<0.001$) in the total reduced glutathione levels in tissues (Viz. gills, muscle, liver, heart and kidney) of *O. mossambicus* subjected to varying sub lethal doses of ethanol during both the exposure periods. Analysis employing ANOVA justifies the above statement (Table 9.3.4a).

Table 9.3.4a Three – Factor ANOVA table for total reduced glutathione

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	1778.279	4	444.570	189.933	0.000
Between Concentrations	402.478	3	134.159	57.317	0.000
Between Days of Exposure	37.824	1	37.824	16.159	0.000
Error	540.693	231	2.341		
Total	2759.274	239			

df- degrees of freedom

Studies done by using Three Factor ANOVA indicated that total reduced glutathione levels varied significantly between tissues ($P<0.001$). There was a significant difference ($P<0.001$) between concentrations. While considering the effect between 7 and 21 days significant difference ($P<0.001$) was noted.

Table 9.3.5 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of conjugated dienes (CD).

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	2.182± 0.0227	2.467± 0.0065	2.642± 0.0631	2.980± 0.1264
	Muscle	1.338± 0.1103	1.979± 0.1189	2.448± 0.0364	2.938± 0.0304
	Liver	1.306± 0.0304	1.532± 0.0348	2.338± 0.1566	2.893± 0.1880
	Heart	1.195± 0.2097	1.753± 0.1239	1.987± 0.0718	2.239± 0.0348
	Kidney	0.796± 0.2240	1.219± 0.0232	1.542± 0.0152	2.477± 0.2561
21 days of Exposure	Gills	2.450± 0.0478	2.441± 0.1316	3.002± 0.0367	3.425± 0.1779
	Muscle	1.127± 0.0740	1.492± 0.0886	2.151± 0.0519	2.856± 0.3952
	Liver	1.108± 0.1218	1.413± 0.0453	2.643± 0.2100	3.174± 0.1674
	Heart	1.058± 0.0022	1.693± 0.0661	2.068± 0.0237	3.220± 0.0352
	Kidney	1.288± 0.0609	1.825± 0.0350	2.035± 0.0152	2.167± 0.0180

Values are expressed as milli moles/100 g wet weight of the tissue.
Average of six values in each group ± SD of six observations.

Figure 9.3.6 Levels of conjugated dienes in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.

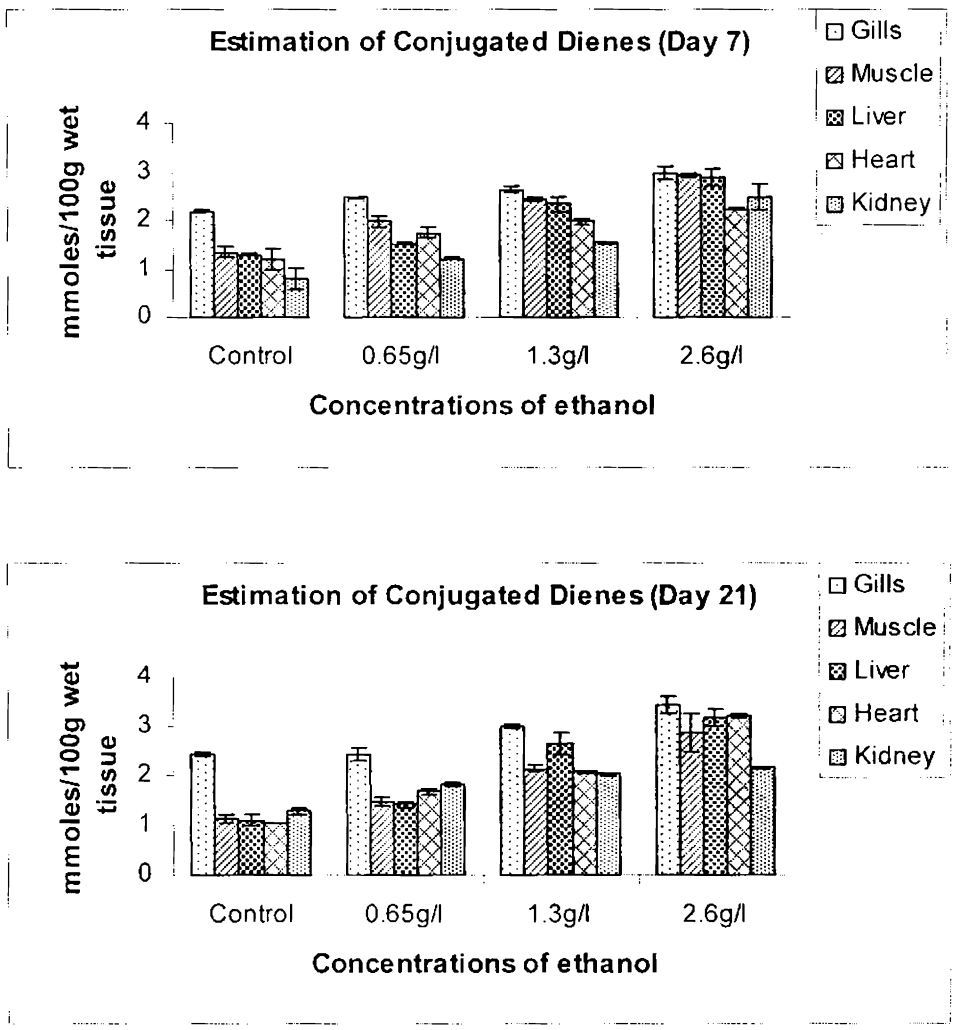


Figure 9.3.6 depicts significant increase ($P<0.001$) in the conjugated diene levels in tissues such as gills, muscle, liver, heart and kidney of *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure period. Analysis carried out by applying ANOVA supports the above statement (Table 9.3.6a).

Table 9.3.6a Three – Factor ANOVA table for conjugated dienes

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	28.117	4	7.029	96.549	0.000
Between Concentrations	71.248	3	23.749	326.205	0.000
Between Days of Exposure	0.851	1	0.851	11.682	0.001
Error	16.818	231	0.073		
Total	117.034	239			

df- degrees of freedom

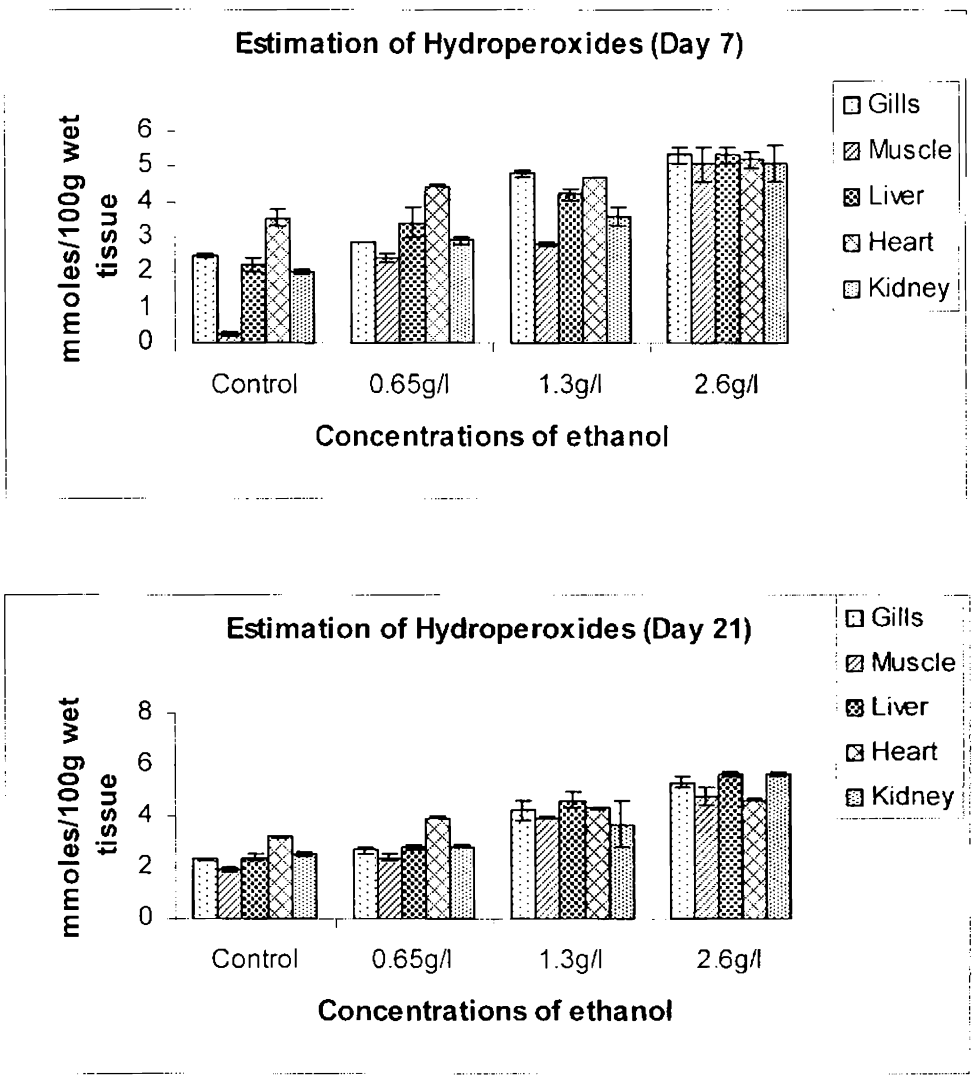
By using Three Factor ANOVA it was noted that conjugated diene level varied significantly between tissues ($P<0.001$). Between concentrations a marked significant difference ($P<0.001$) was noted. After taking into consideration both the 7 and 21 days of exposure period, significant difference ($P<0.001$) was observed.

Table 9.3.7 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of hydroperoxide.

			Concentrations of ethanol		
Days of Exposure	Tissues Analyzed	Control	0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	2.482± 0.0882	2.858± 0.0185	4.805± 0.0819	5.323± 0.2429
	Muscle	0.259± 0.0390	2.403± 0.1116	2.799± 0.0456	5.059± 0.4746
	Liver	2.220± 0.2034	3.418± 0.4508	4.231± 0.1567	5.337± 0.2309
	Heart	3.539± 0.2153	4.462± 0.0186	4.696± 0.0308	5.194± 0.2367
	Kidney	2.024± 0.0835	2.913± 0.0854	3.598± 0.2451	5.086± 0.5022
21 day of Exposure	Gills	2.336± 0.0485	2.660± 0.1072	4.250± 0.3868	5.316± 0.2093
	Muscle	1.893± 0.0890	2.378± 0.1424	3.942± 0.0440	4.760± 0.3291
	Liver	2.364± 0.1166	2.776± 0.0876	4.641± 0.2901	5.658± 0.0895
	Heart	3.186± 0.0729	3.930± 0.0343	4.307± 0.0508	4.652± 0.0362
	Kidney	2.521± 0.0891	2.809± 0.0442	3.684± 0.8963	5.620± 0.0887

Values are expressed as milli moles/100 g wet weight of the tissue.
Average of six values in each group ± SD of six observations.

Figure 9.3.8 Levels of hydroperoxides in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.



Estimation of hydroperoxide level indicated an increase in tissues such as gills, muscle, liver, heart and kidney of *O. mossambicus* (Table 9.3.7 and Figure 9.3.8) and was found to be dependent upon dosage. This was confirmed by using ANOVA (Table 9.3.8a) and the result is mentioned below.

Table 9.3.8a Three – Factor ANOVA table for hydroperoxides

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	44.200	4	11.050	43.231	0.000
Between Concentrations	289.190	3	96.397	377.127	0.000
Between Days of Exposure	0.143	1	0.143	0.561	0.455
Error	59.045	231	0.256		
Total	392.578	239			

df- degrees of freedom

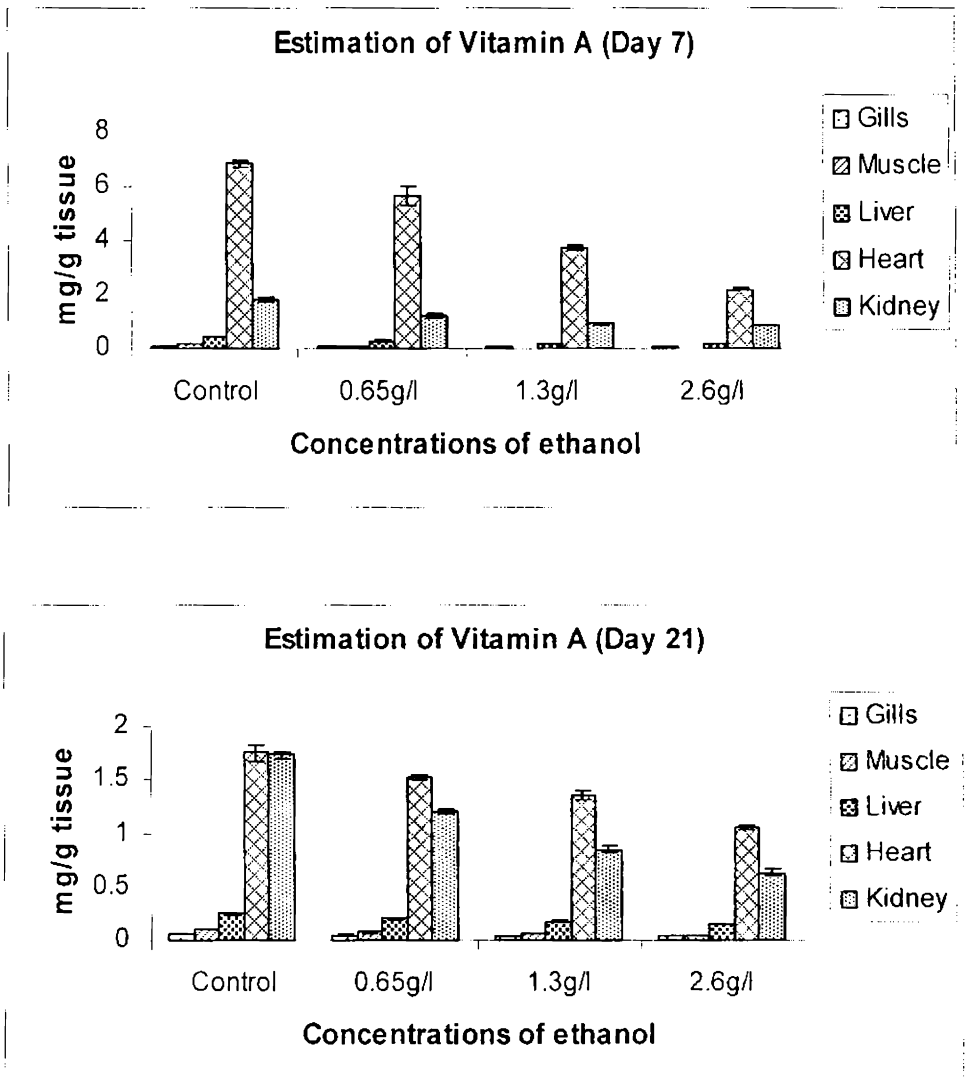
Statistical analysis done by using Three Factor ANOVA revealed that hydroperoxide levels varied significantly between tissues ($P<0.001$). It was noted that between concentrations significant difference was ($P<0.001$). It was concluded by stating that when both the 7 and 21 days of exposure period was taken into account no significant difference (NS) was observed.

Table 9.3.9 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of vitamin A content.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	0.085± 0.0007	0.067± 0.0008	0.063± 0.0007	0.051± 0.0015
	Muscle	0.132± 0.0033	0.061± 0.0019	0.041± 0.0017	0.035± 0.0016
	Liver	0.441± 0.0178	0.299± 0.0288	0.202± 0.0147	0.156± 0.0215
	Heart	6.859± 0.1212	5.637± 0.3297	3.737± 0.0492	2.207± 0.0410
	Kidney	1.803± 0.1007	1.236± 0.0686	0.922± 0.0138	0.865± 0.0259
21 days of Exposure	Gills	0.062± 0.0014	0.054± 0.0008	0.050± 0.0013	0.042± 0.0011
	Muscle	0.101± 0.0027	0.077± 0.0100	0.062± 0.0038	0.035± 0.0023
	Liver	0.248± 0.0085	0.209± 0.0136	0.178± 0.0149	0.152± 0.0020
	Heart	1.758± 0.0711	1.526± 0.0260	1.349± 0.0447	1.052± 0.0249
	Kidney	1.732± 0.0381	1.197± 0.0247	0.842± 0.0327	0.634± 0.0343

Values are expressed as mg/g tissue.
Average of six values in each group ± SD of six observations.

Figure 9.3.10 Levels of vitamin A content in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.



Vitamin A levels were significantly decreased ($P<0.001$) in gills, muscle, liver, heart and kidney tissues of *O. mossambicus* exposed to different sub lethal concentrations of ethanol with respect to control. To validate this ANOVA was carried out and the results are depicted below (Table 9.3.10a).

Table 9.3.10a Three-Factor ANOVA table for Vitamin A

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	306.261	4	76.565	111.155	0.000
Between Concentrations	21.769	3	7.256	10.534	0.000
Between Days of Exposure	27.492	1	27.492	39.912	0.000
Error	159.116	231	0.689		
Total	514.638	239			

df- degrees of freedom

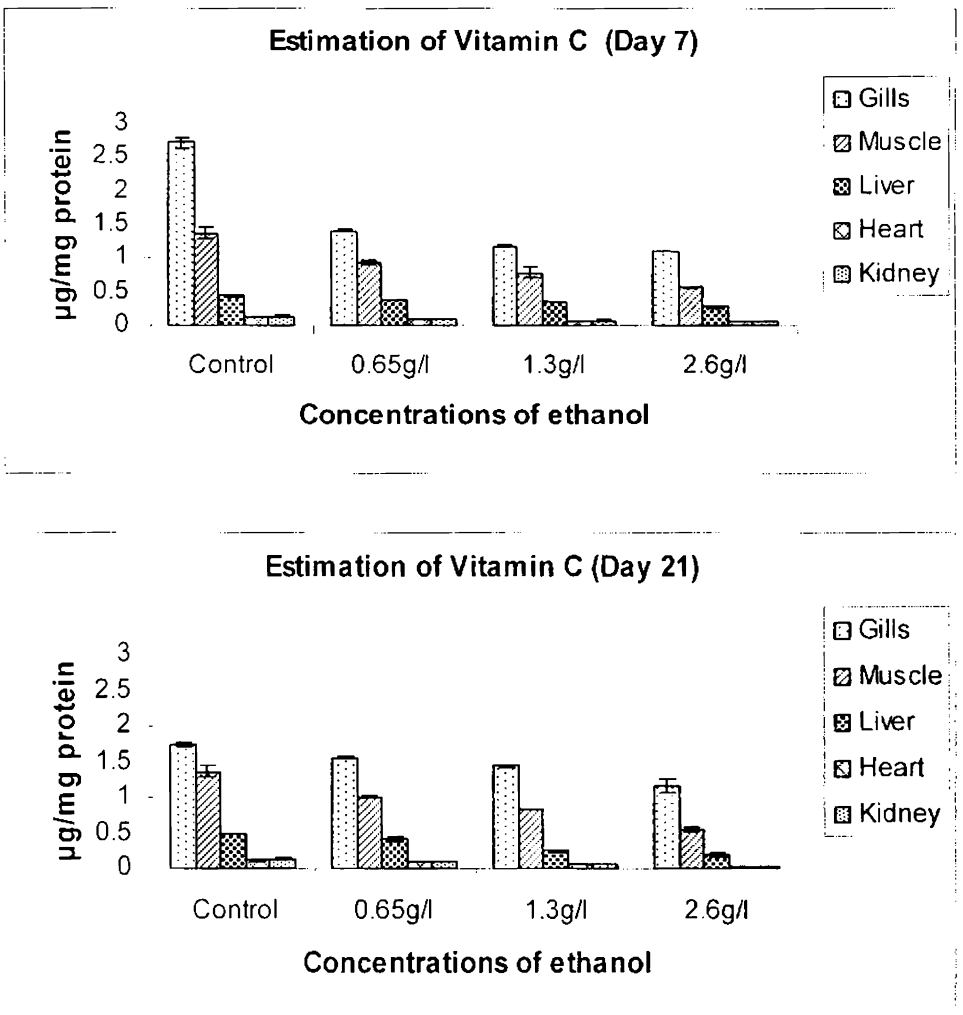
Studies done by using Three Factor ANOVA table exhibited that between tissues significant difference ($P<0.001$) was obtained. When taken into consideration the effect between concentrations significant difference ($P<0.001$) was noted. Also it was concluded by stating that when 7 and 21 days of exposure period was taken into consideration, significant difference obtained was ($P<0.001$).

Table 9.3.11 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of vitamin C content.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	2.738± 0.0825	1.418± 0.0272	1.184± 0.0071	1.119± 0.0056
	Muscle	1.378± 0.0806	0.947± 0.0356	0.798± 0.0677	0.584± 0.0179
	Liver	0.450± 0.0222	0.397± 0.0041	0.361± 0.0118	0.289± 0.0213
	Heart	0.125± 0.0097	0.104± 0.0045	0.066± 0.0025	0.053± 0.0027
	Kidney	0.140± 0.0121	0.091± 0.0027	0.080± 0.0080	0.058± 0.0034
21 days of Exposure	Gills	1.752± 0.0366	1.564± 0.0213	1.442± 0.0150	1.158± 0.1032
	Muscle	1.367± 0.0860	1.012± 0.0162	0.834± 0.0078	0.547± 0.0321
	Liver	0.485± 0.0063	0.414± 0.0329	0.244± 0.0161	0.197± 0.0321
	Heart	0.115± 0.0125	0.100± 0.0024	0.063± 0.0035	0.044± 0.0028
	Kidney	0. 138± 0.0124	0.087± 0.0059	0.064± 0.0016	0.036± 0.0022

Values are expressed as μg/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.12 Levels of vitamin C content in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.



O. mossambicus treated with various ethanol concentrations exhibited significant ($P<0.001$) decrease in ascorbic acid levels in tissues such as gills, muscle, liver, heart and kidney during both periods of exposure (Figure 9.3.12). ANOVA was carried out to ascertain the statement and the table is shown below (Table 9.3.12a).

Table 9.3.12a Three-Factor ANOVA table for Vitamin C

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	76.703	4	19.176	462.881	0.000
Between Concentrations	6.992	3	2.331	56.260	0.000
Between Days of Exposure	0.077	1	0.077	1.851	0.175
Error	9.570	231	0.041		
Total	93.342	239			

df- degrees of freedom

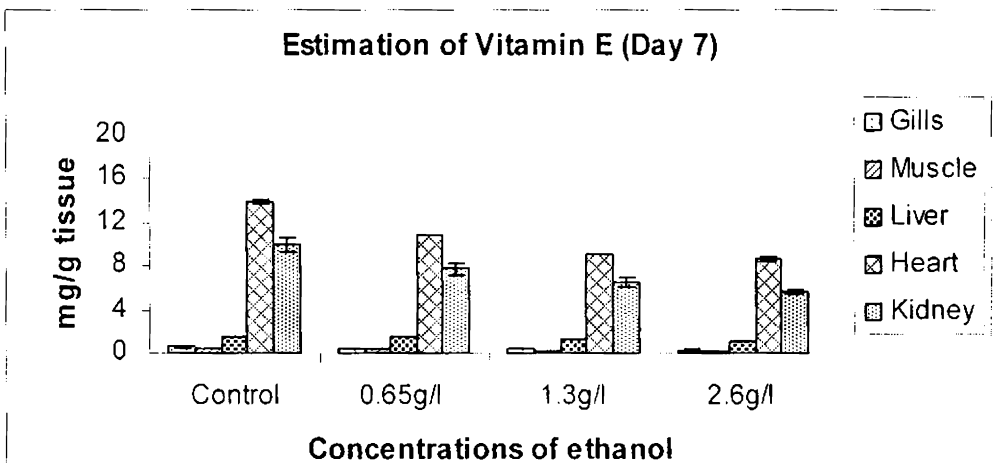
Statistical analysis done by using Three Factor ANOVA table revealed that between tissues and also between concentrations significant difference ($P<0.001$) was obtained. Whereas in the case of effect between days of exposure no significant difference was seen.

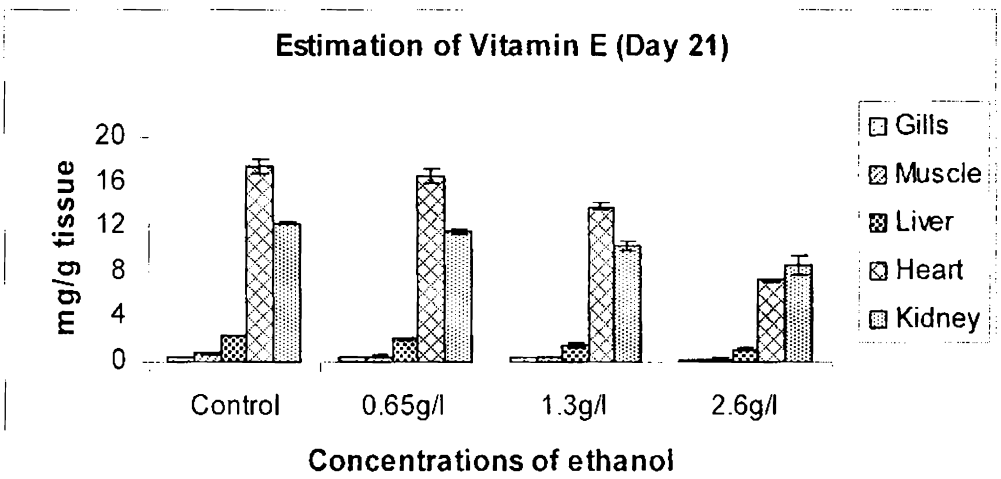
Table 9.3.13 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of vitamin E content

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	0.577± 0.0369	0.433± 0.0087	0.381± 0.0088	0.310± 0.0541
	Muscle	0.520± 0.0173	0.358± 0.0095	0.278± 0.0061	0.222± 0.0122
	Liver	1.526± 0.0241	1.469± 0.0179	1.266± 0.0176	1.034± 0.0515
	Heart	13.92± 0.1846	10.88± 0.0718	9.160± 0.0636	8.689± 0.3078
	Kidney	10.04± 0.6607	7.746± 0.5873	6.474± 0.3974	5.645± 0.1984
21 days of Exposure	Gills	0.407± 0.0163	0.380± 0.0015	0.358± 0.0009	0.266± 0.0304
	Muscle	0.777± 0.0339	0.525± 0.0225	0.436± 0.0111	0.322± 0.0175
	Liver	2.440± 0.0289	2.058± 0.0430	1.485± 0.2335	1.173± 0.1628
	Heart	17.42± 0.6674	16.58± 0.6413	13.86± 0.3761	7.228± 0.0473
	Kidney	12.35± 0.0917	22.59± 0.1832	10.35± 0.3935	8.367± 0.9107

Values are expressed as mg/g tissue.
Average of six values in each group ± SD of six observations.

Figure 9.3.14 Levels of vitamin E content in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.





Significant decrease ($P<0.001$) in vitamin E level (Figure 9.3.14) was observed in the tissues of *O. mossambicus* exposed to all the three sub lethal concentrations of ethanol with respect to control group. To validate this ANOVA was carried out and the results are depicted below (Table 9.3.14a).

Table 9.3.14a Three-Factor ANOVA table for Vitamin E

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	5884.924	4	1471.231	649.201	0.000
Between Concentrations	229.837	3	76.612	33.806	0.000
Between Days of Exposure	115.242	1	115.242	50.852	0.000
Error	523.497	231	2.266		
Total	6753.500	239			

df- degrees of freedom

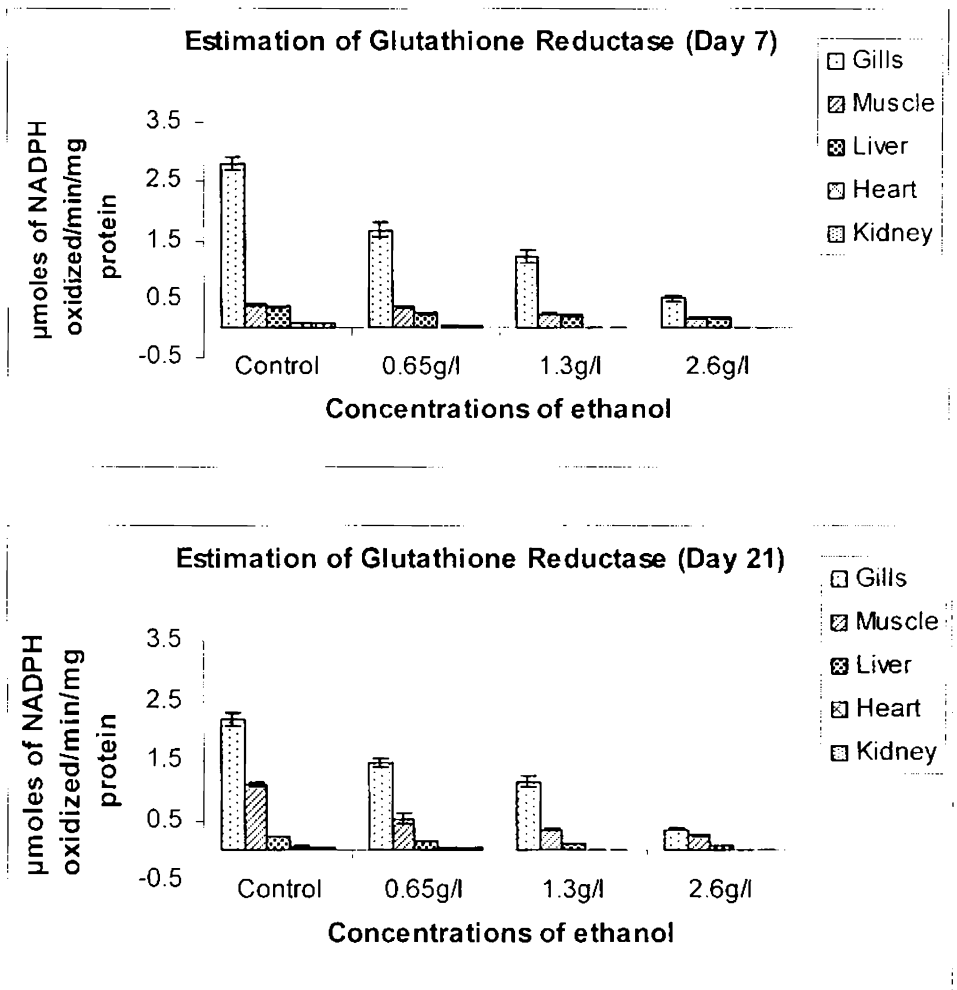
Three Factor ANOVA table stated that significant difference ($P< 0.001$) was obtained when the effect between tissues, between concentrations and also between days of exposure were taken into consideration.

Table 9.3.15 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of glutathione reductase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	2.816± 0.1117	1.676± 0.1287	1.211± 0.1113	0.516± 0.0554
	Muscle	0.411± 0.0169	0.369± 0.0121	0.242± 0.0179	0.178± 0.0170
	Liver	0.372± 0.0226	0.264± 0.0125	0.226± 0.0113	0.182± 0.0203
	Heart	0.100± 0.0029	0.032± 0.0017	0.026± 0.0003	0.023± 0.0003
	Kidney	0.076± 0.0047	0.040± 0.0043	0.024± 0.0008	0.018± 0.0014
21 days of Exposure	Gills	2.189± 0.1031	1.460± 0.0706	1.146± 0.0885	0.350± 0.0224
	Muscle	1.103± 0.0309	0.523± 0.0950	0.351± 0.267	0.246± 0.0303
	Liver	0.216± 0.0017	0.155± 0.0123	0.116± 0.0097	0.091± 0.0023
	Heart	0.061± 0.0044	0.035± 0.0026	0.026± 0.0012	0.012± 0.0006
	Kidney	0. 035± 0.0026	0.033± 0.0023	0.025± 0.0007	0.018± 0.0011

Values are expressed as μmoles of NADPH oxidized/min/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.16 Levels of glutathione reductase in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol



Marked ($P<0.001$) changes were noted in the glutathione reductase values (Figure 9.3.16) in the tissues (gills, muscle, liver, heart and kidney) of *O. mossambicus* subjected to 7 and 21 days of exposure. Analysis using ANOVA authenticates this (Table 9.3.16a).

Table 9.3.16a Three- Factor ANOVA table for glutathione reductase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	64.444	4	16.111	169.106	0.000
Between Concentrations	10.485	3	3.495	36.686	0.000
Between Days of Exposure	0.056	1	0.056	0.589	0.444
Error	22.008	231	0.095		
Total	96.993	239			

df- degrees of freedom

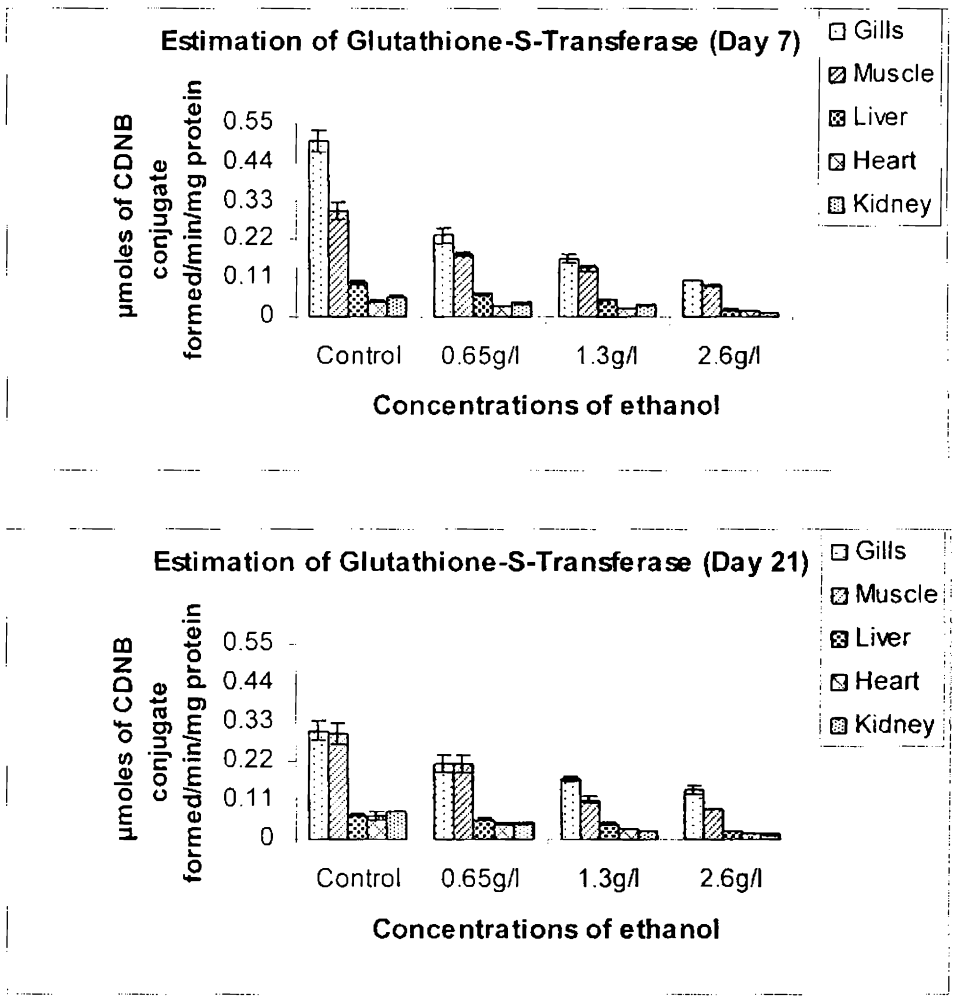
After taking into consideration Three-Factor ANOVA it was observed that between tissues significant difference (P<0.001) was noted. It was inferred that between concentrations also significant difference (P<0.001) was obtained. It was therefore concluded by stating that no significant difference (NS) was obtained when the effect between days of exposure was taken into account.

Table 9.3.17 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of glutathione-S-transferase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	0.502± 0.0314	0.233± 0.0226	0.169± 0.0119	0.106± 0.0018
	Muscle	0.301± 0.0244	0.181± 0.0070	0.141± 0.0087	0.091± 0.0041
	Liver	0.098± 0.0061	0.065± 0.0034	0.051± 0.0003	0.021± 0.0012
	Heart	0.043± 0.0031	0.031± 0.0011	0.027± 0.0009	0.019± 0.0024
	Kidney	0.058± 0.0017	0.040± 0.0015	0.034± 0.0027	0.013± 0.0005
21 days of Exposure	Gills	0.309± 0.0292	0.216± 0.0249	0.171± 0.0053	0.142± 0.0122
	Muscle	0.299± 0.0305	0.215± 0.0262	0.112± 0.0089	0.086± 0.0024
	Liver	0.069± 0.0041	0.058± 0.0014	0.046± 0.0039	0.022± 0.0002
	Heart	0.067± 0.0096	0.048± 0.0031	0.031± 0.0018	0.017± 0.0007
	Kidney	0. 078± 0.0000	0.046± 0.0039	0.025± 0.0012	0.016± 0.0043

Values are expressed as μmoles of CDNB conjugate formed/min/mg protein
Average of six values in each group ± SD of six observations.

Figure 9.3.18 Levels of glutathione-S-transferase in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol



Marked alterations were noted in glutathione-S-transferase levels in tissues (such as gills, muscle, liver, heart and kidney)of *O. mossambicus*, subjected to varying sub lethal concentrations of ethanol during 7 and 21 days (Figure 9.3.18). Investigations using ANOVA corroborates the above statement and the ANOVA table is shown below (Table 9.3.18a)

Table 9.3.18a Three-Factor ANOVA table for glutathione-S-transferase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	1.583	4	0.396	179.810	0.000
Between Concentrations	0.558	3	0.186	84.428	0.000
Between Days of Exposure	0.003	1	0.003	1.537	0.216
Error	0.508	231	0.002		
Total	2.652	239			

df- degrees of freedom

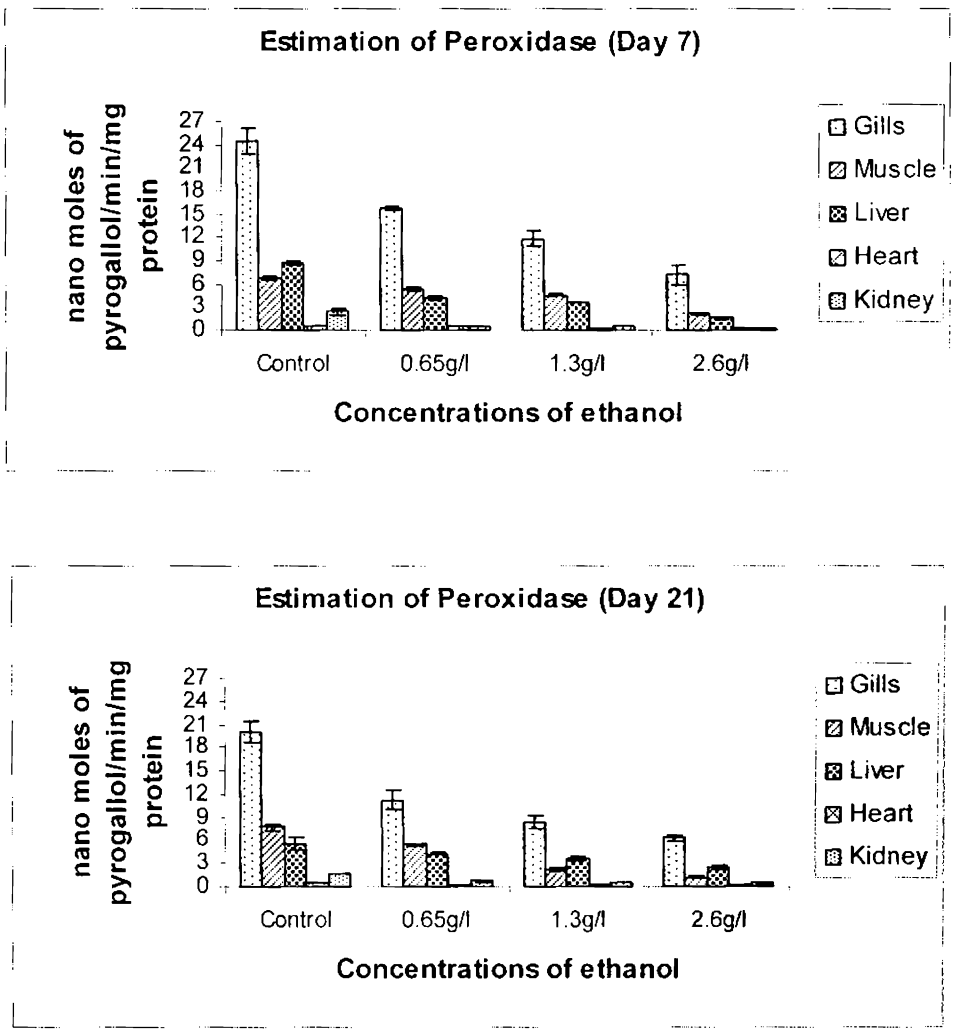
Analysis carried out by using Three Factor ANOVA table indicated that in the case of tissue glutathione-S-transferase when tissues were taken into consideration significant difference (P<0.001) was observed. While taking into consideration the effect between concentrations significant difference (P<0.001) was noted. Finally while considering the exposure between days no significant difference (NS) was observed.

Table 9.3.19 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of peroxidase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol 0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	24.52± 1.7675	15.63± 0.2693	11.92± 0.9784	7.197± 1.3113
	Muscle	6.818± 0.3518	5.369± 0.2385	4.620± 0.1706	2.189± 0.1111
	Liver	8.607± 0.2801	4.325± 0.2634	3.635± 0.1105	1.624± 0.0078
	Heart	0.657± 0.0000	0.558± 0.0277	0.342± 0.0339	0.230± 0.0132
	Kidney	2.418± 0.3783	0.495± 0.0175	0.432± 0.0016	0.349± 0.0056
21 days of Exposure	Gills	20.01± 1.3619	11.22± 1.2499	8.385± 0.7607	6.313± 0.3770
	Muscle	7.657± 0.3105	5.459± 0.2215	2.169± 0.2403	1.224± 0.2427
	Liver	5.666± 0.8339	4.284± 0.1595	3.635± 0.2375	2.479± 0.1758
	Heart	0.591± 0.0758	0.363± 0.0518	0.256± 0.0080	0.237± 0.0096
	Kidney	1.540± 0.0000	0.711± 0.0576	0.555± 0.0287	0.424± 0.0336

Values are expressed as nano moles of pyrogallol /min/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.20 Levels of peroxidase in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol



From the graph it can be inferred that peroxidase level decreased significantly ($P<0.001$) in tissues (such as gills, muscle, liver, heart and kidney) of *O. mossambicus* during immediate and prolonged exposure periods (Figure 9.3.20). Statistical approach implementing ANOVA substantiates this and the table is mentioned below (Table 9.3.20a).

Table 9.3.20a Three-Factor ANOVA table for peroxidase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	5029.808	4	1257.452	247.870	0.000
Between Concentrations	1035.137	3	345.046	68.016	0.000
Between Days of Exposure	52.786	1	52.786	10.405	0.001
Error	1171.868	231	5.073		
Total	7289.599	239			

df- degrees of freedom

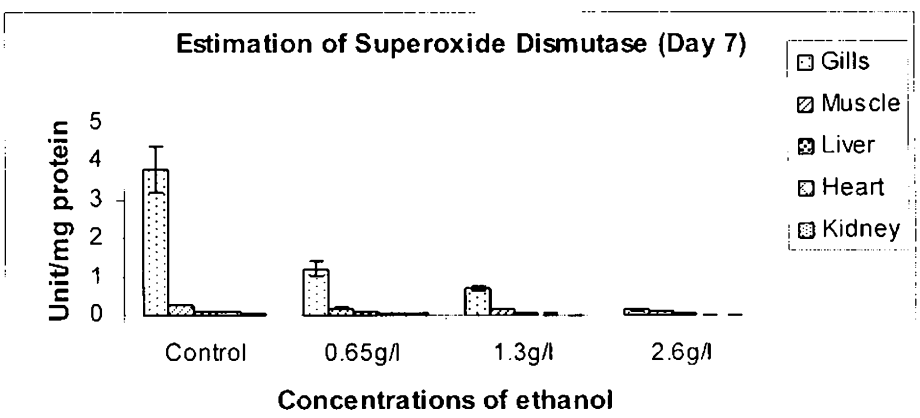
Three factor ANOVA table indicated that while considering the tissue peroxidase levels, between tissues significant difference ($P<0.001$) was observed. When taken the effect between concentrations significant difference ($P<0.001$) was noted. Finally it was concluded by stating that between days of exposure significant difference ($P<0.001$) was obtained.

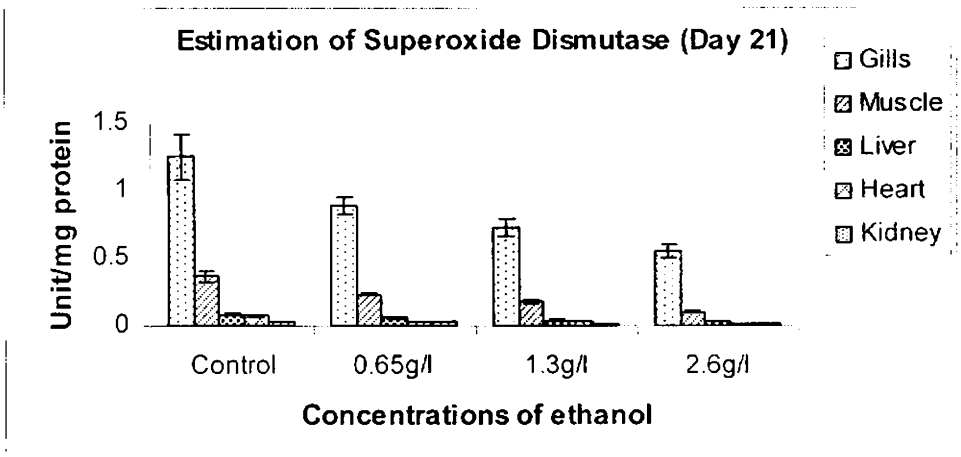
Table 9.3.21 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of superoxide dismutase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	3.803± 0.5841	1.219± 0.2026	0.708± 0.0467	0.154± 0.0241
	Muscle	0.262± 0.0103	0.178± 0.0159	0.149± 0.0057	0.126± 0.0085
	Liver	0.107± 0.0031	0.094± 0.0033	0.075± 0.0026	0.066± 0.0022
	Heart	0.093± 0.0076	0.047± 0.0044	0.026± 0.0017	0.018± 0.0010
	Kidney	0.045± 0.0017	0.037± 0.0034	0.024± 0.0017	0.018± 0.0011
21 days of Exposure	Gills	1.251± 0.1642	0.889± 0.0678	0.728± 0.0626	0544± 0.0515
	Muscle	0.363± 0.0479	0.229± 0.0088	0.178± 0.0129	0.100± 0.0073
	Liver	0.082± 0.0072	0.057± 0.0056	0.038± 0.0038	0.027± 0.0002
	Heart	0.074± 0.0141	0.036± 0.0010	0.028± 0.0006	0.015± 0.0023
	Kidney	0. 035± 0.0024	0.029± 0.0008	0.019± 0.0019	0.013± 0.0022

Values are expressed as units/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.22 Levels of superoxide dismutase in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol





Superoxide dismutase level was found to be significantly decreased ($P<0.001$) (Figure 9.3.22) in tissues (such as gills, muscle, liver, heart and kidney) of *O. mossambicus* when exposed for 7 and 21 days to various sub lethal ethanol concentrations with respect to control. Statistical analysis using ANOVA corroborated the above mentioned statement (Table 9.3.22a).

Table 9.3.22a Three-Factor ANOVA table for superoxide dismutase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	45.504	4	11.376	57.148	0.000
Between Concentrations	8.688	3	2.896	14.548	0.000
Between Days of Exposure	0.949	1	0.949	4.767	0.030
Error	45.983	231	0.199		
Total	101.124	239			

df- degrees of freedom

Statistical interpretation carried out by using Three factor ANOVA exhibited that in the case of tissue superoxide dismutase levels, significant difference ($P<0.001$) was obtained when the effect between tissues, between concentrations were taken into account. When taken into account the effect between days of exposure significant difference ($P<0.05$) was observed.

Table 9.3.23 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of glucose-6-phosphate dehydrogenase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	0.540± 0.0482	0.352± 0.0436	0.148± 0.0133	0.111± 0.0075
	Muscle	0.134± 0.0079	0.120± 0.0147	0.093± 0.0036	0.040± 0.0033
	Liver	0.052± 0.0070	0.031± 0.0015	0.028± 0.0005	0.018± 0.0007
	Heart	0.023± 0.0000	0.018± 0.0004	0.014± 0.0005	0.011± 0.0008
	Kidney	0.037± 0.0000	0.019± 0.0020	0.016± 0.0002	0.013± 0.0000
21 days of Exposure	Gills	0.492± 0.0421	0.366± 0.0299	0.300± 0.0139	0.213± 0.0135
	Muscle	0.111± 0.0073	0.087± 0.0029	0.070± 0.0075	0.042± 0.0082
	Liver	0.036± 0.0015	0.018± 0.0010	0.014± 0.0004	0.011± 0.0015
	Heart	0.023± 0.0024	0.016± 0.0005	0.014± 0.0006	0.008± 0.0012
	Kidney	0. 025± 0.0039	0.017± 0.0000	0.015± 0.0000	0.006± 0.0010

Values are expressed as units/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.24 Levels of glucose-6-phosphate dehydrogenase in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol

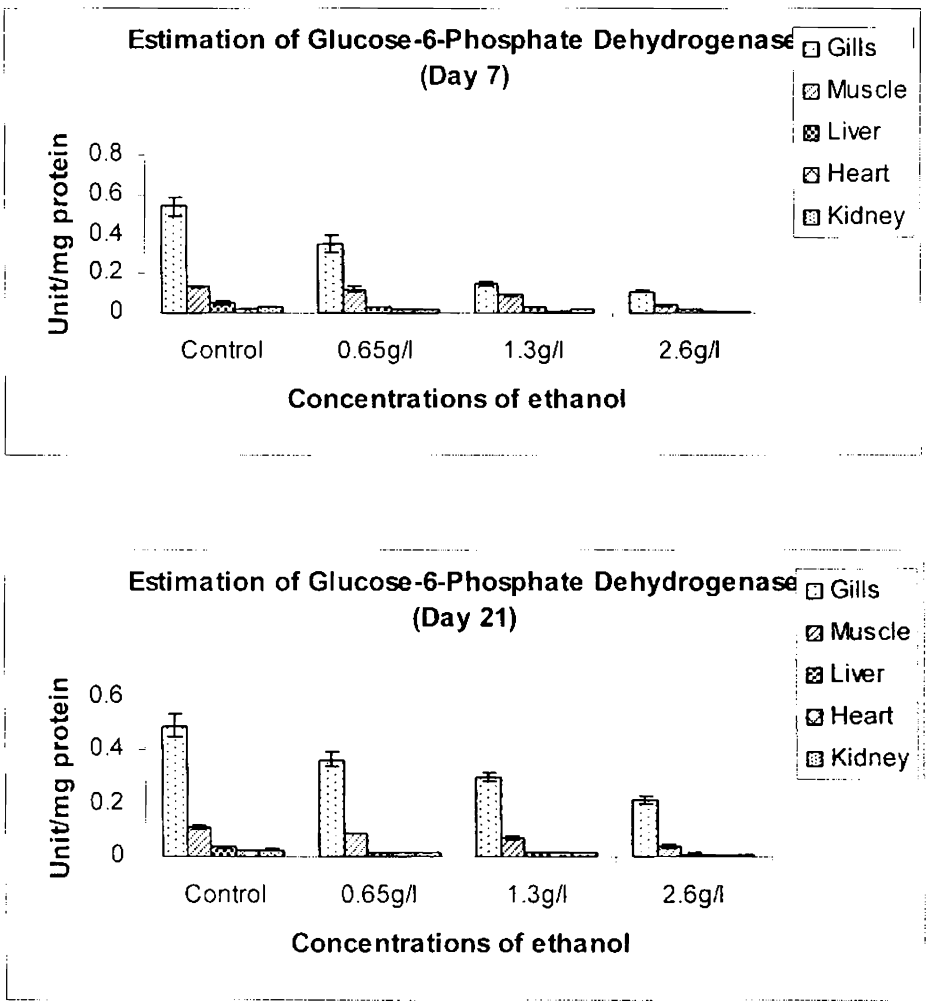


Figure 9.3.24 depicts significant decrease ($P<0.001$) in the glucose-6-phosphate dehydrogenase levels in the tissues such as gills, muscle, liver, heart and kidney of *O. mossambicus* subjected to varying sub lethal concentrations of ethanol during both the exposure periods. Analysis carried out by applying ANOVA supports the above statement (Table 9.3.24a).

Table 9.3.24a Three-Factor ANOVA table for glucose-6-phosphate dehydrogenase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	3.141	4	0.785	238.648	0.000
Between Concentrations	0.339	3	0.113	34.302	0.000
Between Days of Exposure	0.001	1	0.001	0.188	0.665
Error	0.760	231	0.003		
Total	4.241	239			

df- degrees of freedom

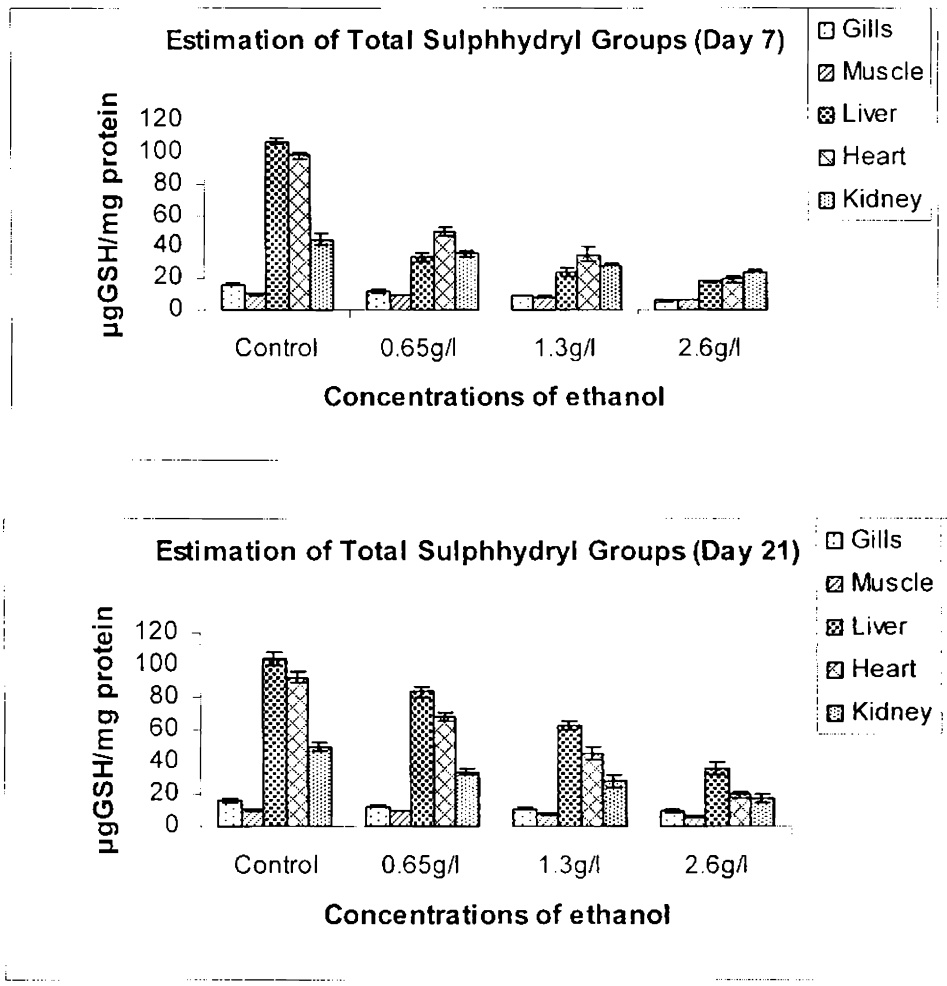
Three Factor ANOVA table revealed that in the case of glucose-6-phosphate dehydrogenase levels between tissues significant difference ($P<0.001$) was observed. Between concentrations there was a significant difference ($P<0.001$). When taken into consideration the effect between days of exposure no significant difference (NS) was got.

Table 9.3.25 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of total sulphhydryl groups.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	16.63± 0.3146	11.94± 1.6200	9.463± 0.4220	6.429± 0.5103
	Muscle	10.41± 0.5112	9.649± 0.2029	8.479± 0.3897	6.526± 0.2204
	Liver	107.0± 2.2707	33.81± 3.1659	24.48± 2.5941	18.37± 0.9176
	Heart	97.94± 1.6338	49.94± 3.1952	35.42± 4.6456	19.71± 2.1608
	Kidney	45.13± 3.5607	35.91± 1.9609	28.82± 0.1998	24.82± 0.8587
21 days of Exposure	Gills	15.73± 1.2542	12.57± 0.3349	11.15± 0.4879	9.316± 1.0530
	Muscle	10.42± 0.4363	9.370± 0.3278	7.788± 0.5353	6.538± 0.5529
	Liver	104.4± 3.8888	83.75± 3.3019	62.61± 2.0740	36.38± 3.7307
	Heart	92.43± 3.2237	68.12± 2.2452	45.54± 4.0863	19.50± 2.0280
	Kidney	48.95± 2.7919	33.98± 2.3807	27.97± 3.8757	17.67± 2.3949

Values are expressed as μg of GSH/mg protein/g tissue.
Average of six values in each group ± SD of six observations.

Figure 9.3.26 Levels of total sulphhydryl groups in the different tissues of *O. mossambicus* exposed for 7 and 21 days to different concentrations of ethanol.



O. mossambicus exposed to varying sub lethal concentrations of ethanol exhibited significant ($P<0.001$) (Figure 9.3.26) decrease in the total sulphhydryl group value in tissues (Viz. gills, muscle, liver, heart and kidney) of *O. mossambicus*. The ANOVA table mentioned below (Table 9.3.26a) justifies this conclusion.

Table 9.3.26a Three-Factor ANOVA table for total sulphhydryl groups

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	102696.540	4	25674.135	123.465	0.000
Between Concentrations	48055.714	3	16018.571	77.032	0.000
Between Days of Exposure	2280.889	1	2280.889	10.969	0.001
Error	48035.819	231	207.947		
Total	201068.962	239			

df- degrees of freedom

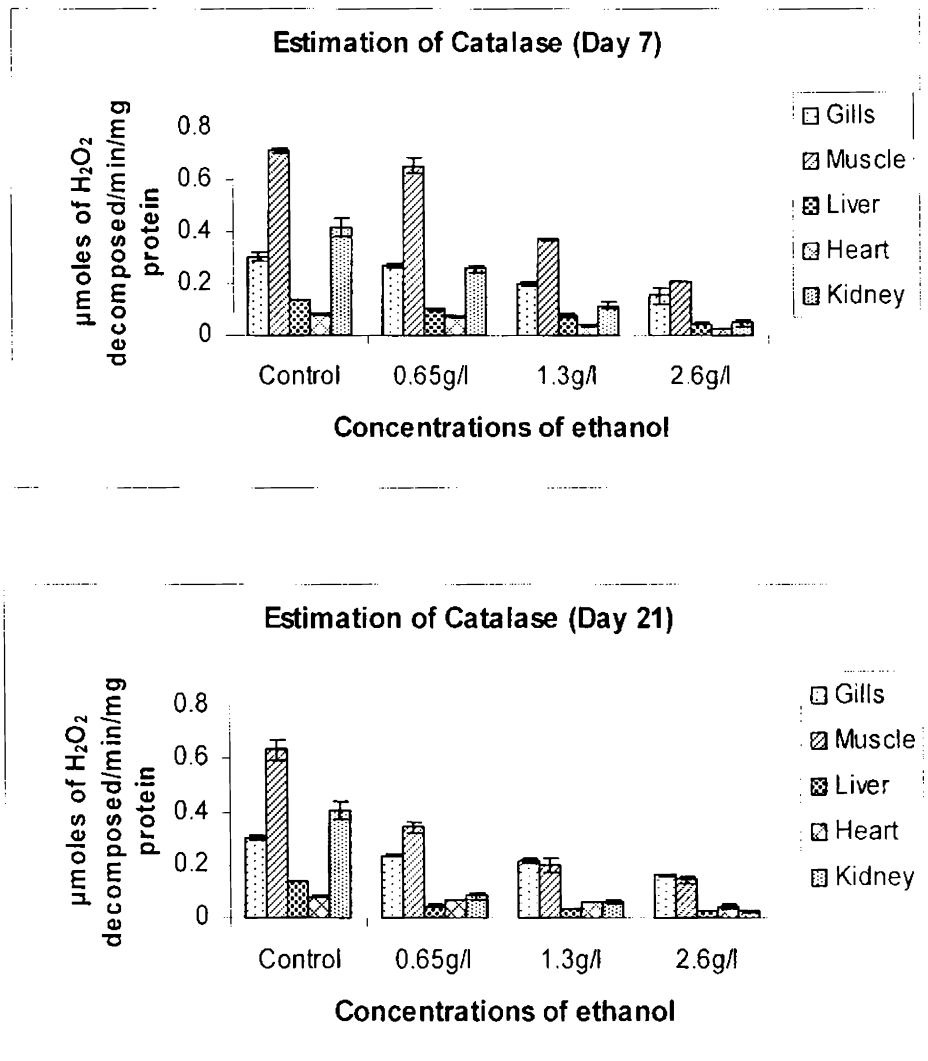
Studies conducted by using Three Factor ANOVA table indicated that in the case of tissue total sulphydryl groups significant difference ($P<0.001$) was obtained when the effect between tissues, between concentrations and between days of exposure, were taken into account.

Table 9.3.27 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of catalase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	0.301± 0.0162	0.269± 0.0063	0.200± 0.0066	0.154± 0.0300
	Muscle	0.715± 0.0091	0.656± 0.0333	0.370± 0.0059	0.207± 0.0000
	Liver	0.138± 0.0022	0.102± 0.0023	0.080± 0.0060	0.045± 0.0037
	Heart	0.085± 0.0056	0.075± 0.0051	0.039± 0.0016	0.027± 0.0024
	Kidney	0.415± 0.0363	0.257± 0.0165	0.117± 0.0162	0.048± 0.0141
21 days of Exposure	Gills	0.301± 0.0058	0.236± 0.0072	0.213± 0.0094	0.160± 0.0065
	Muscle	0.633± 0.0393	0.341± 0.0196	0.201± 0.0249	0.142± 0.0145
	Liver	0.139± 0.0011	0.045± 0.0030	0.033± 0.0022	0.024± 0.0010
	Heart	0.081± 0.0074	0.068± 0.0014	0.061± 0.0006	0.043± 0.0075
	Kidney	0.404± 0.0307	0.083± 0.0115	0.061± 0.0060	0.022± 0.0031

Values are expressed as μmoles of H_2O_2 decomposed/min/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.28 Levels of catalase in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



From the graph (Figure 9.3.28) it can be inferred that catalase levels varied significantly ($P<0.001$) in *O. mossambicus* during immediate and prolonged exposure periods in tissues such as gills, muscle, liver, heart and kidney. Employing ANOVA justifies the above statement (Table 9.3.28a).

Table 9.3.28a Three-Factor ANOVA table for catalase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	3.803	4	0.951	14.831	0.000
Between Concentrations	1.865	3	0.622	92.083	0.000
Between Days of Exposure	0.153	1	0.153	22.614	0.001
Error	1.559	231	0.007		
Total	7.380	239			

df- degrees of freedom

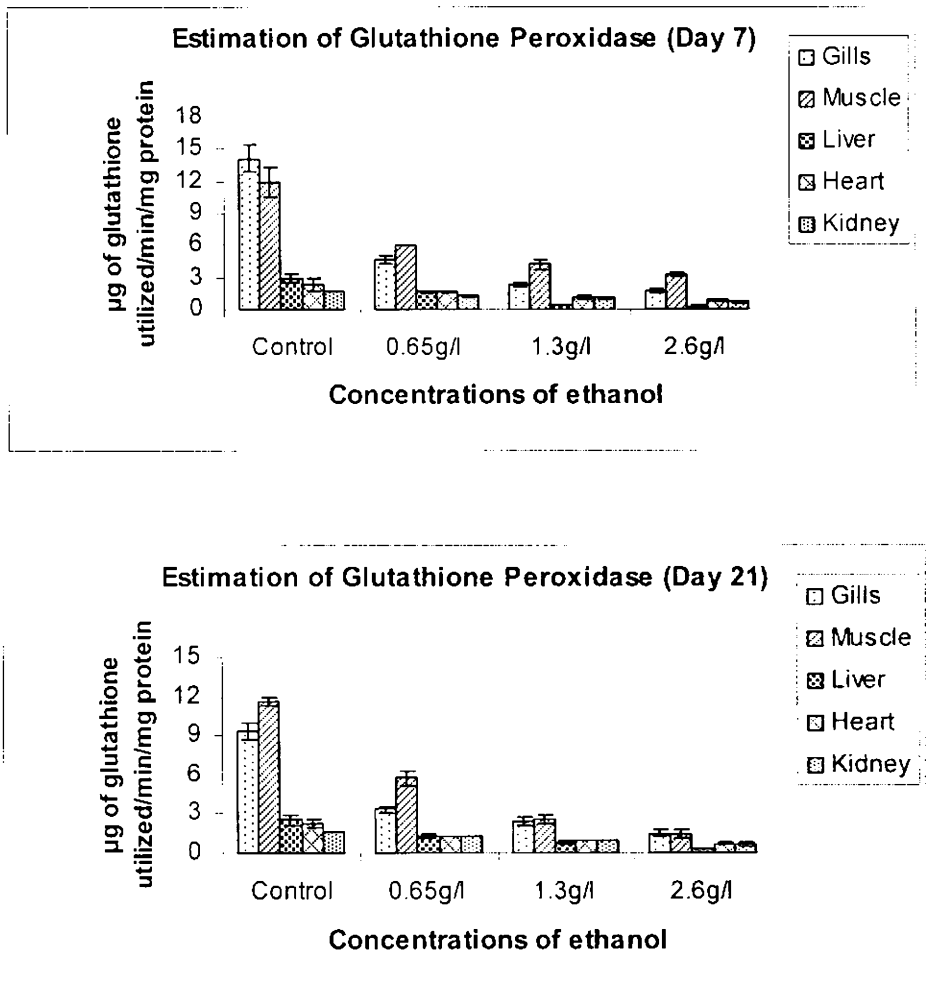
Three Factor ANOVA table indicated that tissue catalase levels varied significantly ($P<0.001$) between tissues. Between concentrations a marked significant difference ($P<0.001$) was noted. Also it was concluded by stating that between days of exposure, significant difference ($P<0.001$) was observed.

Table 9.3.29 Effect of different sub lethal concentrations of ethanol in different tissues of *O. mossambicus* exposed for 7 and 21 days on the level of glutathione peroxidase.

Days of Exposure	Tissues Analyzed	Control	Concentrations of ethanol		
			0.65g/l	1.3g/l	2.6g/l
7 days of Exposure	Gills	14.17± 1.3114	4.722± 0.4371	2.344± 0.1402	1.728± 0.1543
	Muscle	11.91± 1.4046	6.085± 0.0717	4.282± 0.4936	3.3647± 0.1834
	Liver	2.954± 0.3959	1.680± 0.1678	0.423± 0.0290	0.289± 0.0161
	Heart	2.393± 0.5639	1.701± 0.0572	1.225± 0.1570	0.921± 0.0589
	Kidney	1.761± 0.0622	1.293± 0.0645	1.101± 0.0526	0.757± 0.1013
21 days of Exposure	Gills	9.338± 0.5976	3.320± 0.2624	2.378± 0.3302	1.522± 0.2038
	Muscle	11.66± 0.3274	5.782± 0.5421	2.565± 0.3436	1.434± 0.3533
	Liver	2.510± 0.4397	1.226± 0.1581	0.799± 0.1504	0.265± 0.0021
	Heart	2.265± 0.3098	1.309± 0.0435	1.006± 0.0125	0.707± 0.0221
	Kidney	1.599± 0.0125	1.295± 0.0478	0.940± 0.0239	0.595± 0.1578

Values are expressed as μg of glutathione utilized/min/mg protein.
Average of six values in each group ± SD of six observations.

Figure 9.3.30 Levels of glutathione peroxidase in the different tissues of *O. mossambicus* exposed for 7 days and 21 days to different concentrations of ethanol.



Glutathione peroxidase activity was found to be significantly decreased ($P<0.001$) in the tissues of *O. mossambicus* subjected to both durations of exposure to all the three sub lethal concentrations of ethanol (Figure 9.3.30) with respect to control. To validate this ANOVA was carried out and the results obtained are depicted below (Table 9.3.30a).

Table 9.3.30a Three- Factor ANOVA table for glutathione peroxidase

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	1000.767	4	250.192	80.095	0.000
Between Concentrations	864.524	3	288.175	92.255	0.000
Between Days of Exposure	23.752	1	23.752	7.604	0.006
Error	721.569	231	3.124		
Total	2610.612	239			

df- degrees of freedom

Studies conducted by using Three Factor ANOVA table indicates that in the case of tissue glutathione peroxide levels between tissues significant difference ($P<0.001$) was observed. In the case of concentrations significant difference ($P<0.001$) was noted. Also when the effects between days of exposure were taken into account significant difference ($P<0.01$) was obtained.

Table 9.3.31 Multiple Comparison Test (Concentration)

Subsequent comparisons by multiple comparison tests between concentrations using Dunnett's method is shown below.

	Groups	MDA	TRG	CD	HP	Vit.A	Vit.C	Vit.E	GR
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.149[d]	0.000[a]	0.012[c]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.001[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = $P < 0.001$, b = $P < 0.01$, c = $P < 0.05$ and not significant at d.

MDA-Malondialdehyde, TRG-Total Reduced Glutathione, CD-Conjugated Dienes, HP- Hydro peroxides, Vit A-Vitamin A, Vit C-Vitamin C, Vit E-Vitamin E, GR-Glutathione Reductase.

Table 9.3.31 Multiple Comparison Test (Concentration)

Subsequent comparisons by multiple comparison tests between concentrations using Dunnett's method is shown below.

	Groups	GST	POD	SOD	G-6-PD	TSH	CAT	GPx
Dunnett	Control Vs 0.65g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 1.3g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Control Vs 2.6g/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a = P < .0001, b = P < 0.01, c = P < 0.05 and not significant at d.

GST-Glutathione-S-Transferase, POD-Peroxidase, SOD-Super oxide dismutase, G-6-PD- Glucose-6-phosphate dehydrogenase, TSH-Total sulphydryl groups, CAT- Catalase, GPx-Glutathione peroxidase.

A subsequent pair wise comparison in the case of tissue sample between various concentrations with respect to control using Dunnett's method revealed that parameters such as malondialdehyde, total reduced glutathione, conjugated dienes, hydroperoxides, vitamin C, glutathione reductase, glutathione-s-transferase, peroxidase, superoxide dismutase,glucose-6-phosphate dehydrogenase, total sulphhydryl groups, catalase and glutathione peroxidase levels exhibited significant difference (P<0.001). In the case of vitamin A content in different tissues in *O. mossambicus* exposed to ethanol concentration of 0.65 g/l when compared with control exhibited no significant difference ;whereas 1.3 g/l and 2.6 g/l exhibited significant difference (P<0.001). In the case of vitamin E content in different tissues when 0.65 g/l was compared with control significant difference (P<0.05) was obtained whereas when 1.3 g/l and 2.6 g/l was compared with control significant difference (P<0.001) was noted.

Table 9.3.32 Multiple Comparison Test (Tissue)

Subsequent comparisons by multiple comparison tests between tissues using Tukey is shown below.

	Groups	MDA	TRG	CD	HP	Vit.A	Vit.C	Vit. E	GR
Tukey	Gills Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.000[a]	1.000[d]	0.000[a]	1.000[d]	0.000[a]
	Gills Vs Liver	0.000[a]	0.000[a]	0.000[a]	0.945[d]	0.836[d]	0.000[a]	0.002[b]	0.000[a]
	Gills Vs Heart	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Gills Vs Kidney	0.000[a]	0.000[a]	0.000[a]	0.203[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Liver	0.000[a]	0.000[a]	1.000[d]	0.000[a]	0.860[d]	0.000[a]	0.003[b]	0.004[b]
	Muscle Vs Heart	0.003[b]	0.000[a]	0.087[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Kidney	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Liver Vs Heart	0.000[a]	0.275[d]	0.055[d]	0.001[a]	0.000[a]	0.000[a]	0.000[a]	0.075[d]
	Liver Vs Kidney	0.000[a]	0.423[d]	0.000[a]	0.033[c]	0.000[a]	0.000[a]	0.000[a]	0.059[d]
	Heart Vs Kidney	0.000[a]	0.999[d]	0.000[a]	0.000[a]	0.000[a]	1.000[d]	0.000[a]	1.000[d]

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

Table 9.3.32 Multiple Comparison Test (Tissue)

Subsequent comparisons by multiple comparison tests between tissues using Tukey is shown below.

	Groups	GST	POD	SOD	G-6-PD	TSH	CAT	GPx
Tukey	Gills Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.845[d]	0.000[a]	0.070[d]
	Gills Vs Liver	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Gills Vs Heart	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Gills Vs Kidney	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.014[c]	0.000[a]
	Muscle Vs Liver	0.000[a]	0.997[d]	0.611[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Heart	0.000[a]	0.000[a]	0.428[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Muscle Vs Kidney	0.000[a]	0.000[a]	0.335[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	Liver Vs Heart	0.316[d]	0.000[a]	0.999[d]	0.915[d]	0.383[d]	0.883[d]	0.989[d]
	Liver Vs Kidney	0.542[d]	0.000[a]	0.992[d]	0.970[d]	0.000[a]	0.000[a]	0.999[d]
	Heart Vs Kidney	0.996[d]	0.854[d]	1.000[d]	0.999[d]	0.000[a]	0.000[a]	0.942[d]

The values are significant at a = P < 0.001, b = P < 0.01, c = P < 0.05 and not significant at d.

Subsequent pair wise comparison between various concentrations of gill, muscle, liver, heart and kidney tissues of *O. mossambicus* were carried out using Tukey which indicated that in the case of malondialdehyde levels heart tissue when compared with muscle exhibited significant difference (P<0.01) whereas all other possible interactions between tissues exhibited significant difference (P<0.001). In

the case of total reduced glutathione, glutathione reductase, glutathione-S-transferase, glucose-6-phosphate, glutathione peroxidase levels when heart and kidney tissues were compared with liver no significant difference was observed whereas all other possible interactions between tissues exhibited significant difference ($P<0.001$). In the case of conjugated dienes when liver and heart tissues were compared with muscle as well as when heart tissue was compared with liver no significant difference was noted. In the case of hydroperoxides when liver and kidney tissues were compared with gills no significant difference was obtained whereas when kidney tissues were compared with liver significant difference ($P<0.05$) was obtained. In the case of vitamin A levels no significant difference was obtained when muscle and liver tissues were compared with gills, also no significant difference was exhibited when liver was compared with muscle. In the case of vitamin C level no significant difference was obtained when heart tissue was compared with kidney. In the case of vitamin E muscle tissue when compared with gills exhibited no significant difference (NS), whereas liver tissue when compared with gills and muscle exhibited significant difference ($P<0.01$). In the case of peroxidase no significant difference (NS) was observed when liver tissue was compared with muscle similarly heart tissue when compared with kidney also exhibited the same. In the case of superoxide dismutase no significant difference (NS) was obtained when liver, heart and kidney tissues were compared with muscle similarly heart and kidney tissues when being compared with liver also showed no significant difference (NS). Also heart tissue when compared with kidney also exhibited no significant difference (NS). In the case of total sulphydryl groups and glutathione peroxidase no significant difference was seen when heart tissue was compared with liver.

9.4D Discussion

Fish is referred to as extremely sensitive bioindicator of aquatic pollution and is been preferred as a test species in toxicological screening of water. Gills are the first organs to be exposed to water-borne contaminants (Gallagher and Di Giulio, 1992). Kidney plays a vital role in the maintenance of an organism's internal environment, being the key organ involved in the regulation of extracellular fluid

volume and composition as well as acid–base balance. It is also a target of toxic chemicals, which can disrupt its functions, and cause temporary or permanent derangement of homeostasis (Miller, 2002). Liver has a pivotal role in the regulation of physiological processes. It is involved in several vital functions such as metabolism, secretion and storage. Detoxification of a variety of xenobiotics occurs in liver. The oxidative stress is induced by the production of reactive oxygen species (ROS) (Lemaire *et al.*, 1996). Reactive oxidative species are continuously produced in physiologically active aerobic organisms. When the rate of ROS formation is excessive it can overwhelm the antioxidant capacity of organisms creating oxidative stress (Sies, 1986). McCarthy and Shugart (1990) suggested that oxidative stress biomarkers could be employed in environmental monitoring programmes. Free radical scavenging enzymes such as SOD, CAT, GPx and GST are the first line of defense against oxidative injury. The inhibition of antioxidant system may cause the accumulation of H_2O_2 or products of its decomposition (Halliwell, 1994).

There is also increasing evidence stating that alcohol toxicity is associated with an increased oxidative stress and free radical associated injury (Cederbaum *et al.* 1989). Generation of oxygen metabolites such as superoxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) is believed to be important in the pathogenesis of alcoholic liver injury (Thurman and Handler, 1989). To counteract these oxidants, cells have several antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Reactive Oxygen Species (ROS) are generated from leakage of electrons onto oxygen from mitochondrial electron transport chain, microsomal cytochrome P_{450} and their electron donating enzymes and other systems. Inactivation and removal of ROS depend on reactions involving the antioxidative defense system. The endogenous antioxidant defense includes enzymatic (e.g. SOD, CAT, POD etc.) and non-enzymatic (e.g. Vit C, Vit E, GSH etc.) systems.

Damage brought about to the tissues in *O. mossambicus* is mediated through the action of toxic oxygen radicals generated by ethanol. The findings stated by Zhao *et al.* (1996) and De la Maza *et al.* (1995) supported the present study. It has been suggested that free radical intermediates produced during ethanol metabolism

might be responsible for causing oxidative damage (Clot *et al.*, 1994). Reports of Rouach *et al.* (1997) indicated that ethanol impairs the antioxidant potential capacity in tissues. It has also been pointed out that lipoperoxidation, a degradative process affecting membraneous polyunsaturated fatty acids, has been suggested to represent an important mechanism of ethanol induced toxicity. It is suggested that the disturbances of iron metabolism reported earlier in human alcoholics (Nordmann and Rouach, 1987) may contribute to an enhanced steady state concentration of reactive free radicals leading to lipoperoxidative damage and cellular injury, in fish also.

Lipid peroxidation is a complex and natural deleterious process. An increase in the level of lipid peroxidation is the evidence most frequently cited in support of the involvement of oxidative stress in tissues (Halliwell 1989, 1992; Liu and Mori 1994). Exposure to polluted water induced the tissue-specific peroxidative damage in gill, kidney and liver of *A. anguilla* (Ahmad *et al.*, 2004). An increase in the MDA level observed in the tissues of *O. mossambicus* exposed to ethanol for 7 and 21 days (Table 9.3.1 and in Figure 9.3.2). This mainly arose due to the inactivation of antioxidant system. The increase in tissue MDA is due to the increased generation of reactive oxygen species (ROS) due to the excessive oxidative damage resulting in the production of free radicals. Another possible reason could be due to the increased activity of reduced nicotinamide-adenine dinucleotidephosphate (NADPH) oxidase which may contribute to enhanced lipid peroxidation. This was supported by Panneerselvam and Devadoss (1998) who also observed similar increase in lipid peroxidation in the liver and kidney of rats when treated with Auramine O for 7 days. Also in the present study, the levels of LPO and conjugated dienes increased significantly in *O. mossambicus* intoxicated with ethanol. Similarly a significant increase in lipid peroxidation level in tissues was observed when *O. mossambicus* was exposed to ethanol for 21 days. This could be due to the decreased levels of antioxidants and antioxidant enzymes. The observed abnormalities in the tissues of fishes may be in part due to heperlipidemia leading to changes in the activity of antiperoxidative enzymes, glutathione and increased lipid peroxidation. This was supported by Mahendran and Shyamala Devi (2001) who also observed similar increase in lipid peroxidation on ethanol induced peroxidative damage in rats

when exposed for 45 days. Nordmann *et al.* (1992) revealed that the chronic administration of ethanol increased the levels of LPO, decreased the activities of SOD and CAT and reduced the content of GSH. Nalini *et al.* (2003) observed similar increase in the MDA levels when treated with ethanol for 25 days in swiss albino mice. Achuba and Osakwe (2003) also observed similar increase in lipid peroxidation level in the muscle, liver, kidney, heart, gills and intestinal tract of African catfish, *C. gariepinus* exposed to various doses of oil in water dispersions of Nigerian petroleum for 0, 7, 14, 21 and 28 days.

SOD is an ubiquitous chain breaking antioxidant and is found in all aerobic organisms. In the present study a decrease in superoxide dismutase activity was found when *O. mossambicus* was subjected to sub chronic ethanol exposure for 7 and 21 days (Table 9.3.21 and Figure 9.3.22). SOD catalyzes the conversion of superoxide anion into H_2O_2. The inhibition of SOD brought about by ethanol may be due to the interaction directly with superoxide molecules. SOD is a metallo enzyme, depending on its sub cellular origin, containing cu/Zn in its structure. It is possible that ethanol interacts with these metal moieties and produces inhibition in enzyme activity. Reduction in SOD activity results in an increased peroxidative damage in the tissues which is evident in the present investigation. This was supported by Panneerselvam and Devadoss (1998) who noticed similar changes in the liver and kidney of rats treated with Auramine O for 7 days. Two antiperoxidative enzymes namely SOD and CAT decreased significantly in the hepatic tissues of alcohol administered rats suggesting that the increased damage to this tissue may be the result of uncontrolled generation of partially reduced oxygen species. SOD inhibition by ethanol reflects either a large scale utilization of the enzyme or a decrease of its synthesis. Decreased activity of superoxide dismutase observed in the tissues of *O. mossambicus* mainly points to the excessive generation of $O_2^{\cdot-}$ leading to inactivation of the enzyme. Also, increased reactive oxygen species, partly generated from acetaldehyde oxidation, may contribute to the occurrence of oxidative stress. The deleterious impact brought to the tissues by producing oxidative stress was also evidenced in histological changes in the present study.

In the present study a decrease in catalase activity was found when *O. mossambicus* was subjected to sub chronic ethanol exposure for 7 and 21 days (Table 9.3.27 and Figure 9.3.28). Catalase found mainly in the peroxisomes, removes H_2O_2 produced during oxidations. In the present study inhibition of Catalase activity observed in the tissues of *O. mossambicus* following ethanol exposure may therefore have an important role in enhancing oxidative stress of cellular system. Catalase is an efficient inhibitor of LPO when hydrogen peroxide accumulates in a cell containing free ferrous ions. The primary role of catalase is to scavenge H_2O_2 that has been generated by free radical. The activity of antioxidant enzyme catalase which is responsible for the detoxification of significant amounts of hydrogen peroxide is found to be decreased in the present study on exposure to ethanol. The increased H_2O_2 level augments the risk of oxidative damages to tissues. Catalase catalyses the dismutation of hydrogen peroxide. Decreased catalase activity may be due to loss of NADPH, or generation of superoxide, or increased activity of lipid peroxidation or combination of all upon chronic exposure to ethanol.

GPx works in tandem with catalase to scavenge excess H_2O_2 as well as lipid peroxidation in response to oxidative stress (Kabuto *et al.*, 2003). In the present study a decrease in glutathione peroxidase activity was found when *O. mossambicus* was subjected to sub chronic ethanol exposure for 7 and 21 days (Table 9.3.29 and Figure 9.3.30). GSH-Px another antiperoxidative enzyme is present both in cytosol and in mitochondrial matrix. Ethanol inhibits the enzyme directly by impairing the functional groups or indirectly by reducing the supply of reduced glutathione (GSH) and NADPH needed for its action. Ethanol induced decrease in GSH-Px activity may arise as a consequence of selenium mediated detoxification when the selenium concentration runs short to maintain both GSH-Px activity and detoxification at an optimal level. The decrease in GPX observed during ethanol toxicity studies may be due to their increased utilization to scavenge the significantly elevated levels of ROS that has been formed. The non availability of glutathione decreases the activity of glutathione peroxidase and glutathione transferase. Another reason for the decrease in GPx activity could be the inhibitory effect on protein synthesis brought about by ethanol and/or increased ROS production. Glutathione peoxidase reduces hydrogen peroxide and organic hydroperoxides and thus protects against free radical damage.

Panneerselvam and Devadoss (1998) found similar decrease in the GPx activity in the liver, kidney of rats when treated with Auramine O for 7 days. Decrease in the activity of GPx observed during prolonged exposure in the present investigation may be due to exhaustion or inactivation of the enzyme by reactive oxygen species.

The GSTs are a multigene family of isozymes that catalyze the conjugation of GSH to a variety of electrophilic compounds, and thereby exert a critical role in cellular protection against ROS (Hayes and Pulford, 1995; Wilce and Parker, 1994). Thus, ethanol or its metabolic products might specifically target GST isoenzymes and the reduction in enzyme activity or expression may contribute to ethanol hepatotoxicity (Alin *et al.*, 1985). A significant decrease in glutathione–S-transferase activity was found when *O. mossambicus* was exposed to sub chronic ethanol for 7 and 21 days (Table 9.3.17 and Figure 9.3.18). GST, an antioxidant enzyme, provides protection to the tissues by catalyzing the conjugation of a variety of electrophilic xenobiotics to GSH (Chasseaud, 1979). GST has thiol group which is mainly responsible for the inhibition in GST activity. The decrease in GST observed during ethanol toxicity studies may be due to their increased utilization to scavenge the ROS formed in significantly elevated levels.

When fishes were exposed to environmental stress, alterations in the activity of (NADPH) were observed (Saxena *et al.*, 1982; Sastry and Rao, 1984). The studies of Schultz and Harman (1980) reveal that toxic substances commonly used in the field of fisheries caused a significant inhibition of G-6-PD. The findings of Gonzalez and Tejedor (1993), supports this by stating that xenobiotic chemicals inhibit G-6-PD and consequently decrease the reducing power of the cells, affecting cellular biosynthetic functions. G-6-PD catalyses the conversion of β-D-glucose-6-phosphate to 6-phosphoglucono- δ-lactone in the pentose phosphate pathway and this reaction is the major step for generation of reduced potential (NADPH). Gupta (1987) reported inhibition of the enzyme resulting in the decreased biosynthetic and detoxification mechanism of the cells in *Channa punctatus* exposed to vegetable oil factory effluent. GR inhibition might reflect a possible antioxidant defense failure leading to reported increase in lipid peroxidation. In fish G6PD might play such a crucial role in maintaining the redox state of the cell and in modulating the

antioxidant defenses through the control of NADPH generation. The lower availability of NADPH would render a reduced GR activity, which would impair GSH regeneration from GSSG. The decrease observed in glutathione reductase activity (Table 9.3.15 and figure 9.3.16) as well as in glucose-6-phosphate dehydrogenase activity (Table 9.3.23 and in figure 9.3.24) mainly points out that these enzymes are important in maintaining glutathione in the redox state. The decrease in G-6-PD suggests decreased supply of NADPH for catalytic activity of GR, consequently there was a fall in the GR activity. The decrease in all the enzymes indicates severe impairment and synthesis of the enzymes during ethanol treatment. Oh *et al.* (1998) reported significant decrease in glucose-6-phosphate dehydrogenase activity in ethanol treated groups compared to control.

Raju Ilavarasan *et al.* (2003) observed an increase in lipid peroxidation level followed by a significant decrease in GPx, SOD, GST, GR, CAT in the liver tissues of male Wistar rats when subjected to carbon tetrachloride induced toxicity for 7 days. Mary and Reddy (1999) also found similar trend in the kidney of swiss albino mice when exposed to lead nitrate for 7 and 15 days. An increase in the MDA level in gills followed by decrease in the SOD, GPx and CAT activity during immediate exposure to ethanol was observed by Wang *et al.*, (2006) based on his observations in the mud crab *Scylla serrata* subjected to cold stress. A similar decrease in SOD and CAT activity in the liver and kidney tissues of rats treated with ethanol for 45 days as reported by Nalini *et al.* (2003) supports the present study. The reduced activity of CAT and SOD in the presence of ethanol may cause the accumulation of O_2^-, H_2O_2 or the products of its decomposition. Loss of CAT and SOD activity results in oxygen intolerance and triggers a number of deleterious reactions.

GSH is presumed to be an important endogenous defense against the peroxidative destruction of cellular membranes. GSH can either detoxify activated oxygen species such as H_2O_2 or it can reduce lipid peroxides themselves. Tissue GSH concentration reflects the potential for detoxification. Reduced glutathione which is synthesized mainly in the liver serves as an important non enzymatic antioxidant in the antioxidative defence system. The marked depletion of GSH observed in the tissues intoxicated with ethanol revealed the severity of oxidative

stress. Another possible reason could be the low GSH/GSSG ratio. A decrease in reduced glutathione level (Table 9.3.3 and Figure 9.3.4) observed in the tissues of *O. mossambicus* when exposed to ethanol may be related to the increased lipid peroxidation. Liver glutathione after alcohol administration was found to decrease due to increased utilization by the hepatocytes because GSH seems to act as scavenger for toxic chemical agents. Ethanol-induced depletion of GSH in the tissues of *O. mossambicus* has been cited as evidence supporting the hypothesis that reactive oxygen intermediates generated during the metabolism of ethanol leads to glutathione oxidation and lipid peroxidation. Lowered level of GSH represents an increased utilization of GSH for the activity of glutathione peroxidase forming oxidized glutathione (GSSG) due to oxidative stress. Panneerselvam and Devadoss (1998) found similar decrease in the GSH levels in the liver and kidney of rats when treated with Auramine O for 7 days. A decrease in glutathione was observed in climbing perch (*A. testudineus*) exposed to industrial pollutants after short- and long-term exposure (Chatterjee and Bhattacharya, 1984), in the liver of bullhead (*Cottus gobio* L.) from a polluted site (Bucher *et al.*, 1993) and in channel catfish exposed to bleached kraft mill effluents (Mather and DiGiulio, 1991). Decreased GR activity may be a predominant cause for GSH depletion observed in *O. mossambicus* upon prolonged exposure to ethanol.

A significant decrease ($P<0.001$) (Table 9.3.11 and figure 9.3.12) in ascorbic acid level was observed in the tissues of *O. mossambicus* when exposed to ethanol for 7 and 21 days. The decrease in vitamin C could be due to oxidative stress. There is increased generation of oxygen free radicals and depletion of endogeneous antioxidants. Significantly lowered levels of vitamin C could be due to its enhanced consumption due to increased oxidant stress caused by free radicals. Another reason could be the decrease in catecholamine release from the adrenal medulla (where both are stored). This counteracts the fall in vitamin C owing to oxidative stress. Ascorbic acid is synthesized from glucose and the decrease in the tissue glucose level may be a factor for the reduction of ascorbic acid level in fish. Agrawal *et al.* (1987) described the depletion of ascorbic acid in fishes under organochlorine stress. The findings are also in accordance with that of Kumar and Pandey (1981) and

Nanda and Behera (1996). Subir and Vasudevan (2005) also observed similar decrease in vitamin C level when treated with ethanol for 4 weeks in rats.

A significant decrease ($P<0.001$) (Table 9.3.9 and Figure 9.3.10) in vitamin E level was observed in the tissues of *O. mossambicus* when exposed to ethanol for 7 and 21 days. The decrease in vitamin E refers to the basis of consumption of the membrane free radical scavenger in presence of excess oxygen free radicals formed during ethanol induced toxicity. Another possible reason could be that the increased free radical generation helps in resuming blood flow which results in increased consumption of vitamin E. The decrease in the non enzymatic antioxidant parameters such as GSH, vitamin C and vitamin E may be due to the increased turnover, for preventing oxidative damage suggesting an increased defense against oxidant damage. Similar results were exhibited by Reema *et al.* (2007) who also found decrease in vitamin C and vitamin E levels in the heart tissues followed by acute myocardial infarction.

In the present study a decrease in total SH groups were obtained in the tissues of *O. mossambicus* when exposed to ethanol for 7 and 21 days as indicated in Table 9.3.25 and in Figure 9.3.26. The decrease in total SH groups is mainly arises due to the decrease in membrane ATPase activity. The loss of total SH content might be responsible for the low level of enzyme activities. Similar decrease in total SH content was observed in cirrhotic patients by Surendran *et al.* (2007).

A decrease in vitamin A level was observed in the tissues of *O. mossambicus* when exposed for 7 and 21days. The decrease in vitamin A level observed during immediate exposure could be mainly due to increased serum retinyl esters in lipoproteins. This was supported by Mayumi *et al.* (1982). Similarly decrease in vitamin A observed in all the tissues of *O. mossambicus* when exposed for 21 days could be due to the increased mobilization of vitamin A from the liver, and increased catabolism of vitamin A in the liver or in other organs Mayomi Sato and Charles (1981). It has been reported that metabolism of retinoic acid (a physiological metabolite of vitamin A) proceeds through a microsomal cytochrome P- 450 dependent enzyme system. This metabolism is postulated to

represent the initial degradative step for the elimination of retinoic acid from the body. Chronic ethanol consumption has been shown to induce a variety of hepatic microsomal cytochrome P-450 mediated enzyme activities. Tuchweber *et al.* (1976) have reported that chronic administration of microsomal enzyme inducers decreases hepatic vitamin A in hypervitaminotic A rats. Therefore, it is conceivable that ethanol consumption similarly decreases hepatic vitamin A through increased metabolism of retinoic acid in the liver. Moreover, increased catabolism of vitamin A outside the liver should also be considered. One possible mechanism for the effect of ethanol could be increased release of lipoprotein-bound retinyl esters from the liver into the circulation, but at present, there is no proof that the liver can release lipoprotein-bound retinyl esters. Another possible mechanism is that retinyl esters formed in peripheral tissues are not taken up well by the liver after an acute dose of ethanol. In conclusion, an acute dose of ethanol decreases hepatic vitamin A and increases serum vitamin A, most likely because of increased release from the liver or decreased uptake by the liver of retinyl esters as part of the lipoproteins. This effect may contribute to the depletion of hepatic vitamin A after chronic ethanol consumption. A decrease in vitamin A as well as vitamin C was found in the case of alcoholic liver disease by Yngve *et al.* (2000).

In the present study a significant increase ($P<0.001$) in conjugated dienes (Table 9.3.5 and Figure 9.3.6) and hydroperoxides (Table 9.3.7 and Figure 9.3.8) was observed in the tissues of *O. mossambicus* when exposed to ethanol for 7 and 21 days. Conjugated Dienes are polyunsaturated molecules having alternate double bonds. They are formed at the onset of lipid peroxidation when polyunsaturated fatty acids are attacked by oxygen centered free radicals. They are also linked to several steps of lipid peroxide degeneration. It is reported that about 30-35% of lipid peroxidation is actually detected by diene measurements. Lipid hydroperoxides (LHP) are measured by their ability to oxidize ferrous ion to ferric ion which depends not only on the rate of initiation of peroxidation but also their decomposition to other products. LP markers comprised diene conjugates (DC), LOOH, thiobarbituric acid (TBA), and TBA reactive substances. The free radical attack on cell membrane-bound polyunsaturated fatty acids results in formation of LP products such as DC, LOOH, and malondialdehyde. High levels of DC, LOOH,

and malondialdehyde (the latter is expressed as TBA activity) are considered to be markers of systemic oxidative stress Halliwell and Gutteridge (1999). The increase in LPO,CD and HP as well as decrease in T-SH, Vit C and Vit E were supported by the findings of Panchamoorthy and Carani (2007) observed similar increase in LPO, CD and HP as well as decrease in TSH, Vit C and Vit E, in the skeletal muscle of rats when treated with L-carnitine for 30 days.

The decrease in peroxidase activity observed in the tissues of *O. mossambicus* when exposed to 7 and 21 days mainly points to inhibition of enzyme synthesis. This supports the findings of Charles *et al.* (1998). Peroxidase activity might be expected to reduce the level of ROS by metabolizing H_2O_2. Peroxidase is also capable of various "oxidase" reactions leading to H_2O_2 generation.

In conclusion this study clearly indicates that significant ($P<0.001$) alterations exhibited by both non enzymatic and enzymatic antioxidant parameters discussed in this section can be employed as biomarkers in aquatic system, brought about by ethanol spillage incidents in the nearby future. Pedrajas *et al.* (1996) and Winston and Di Giulio (1991) observed that the exposure to xenobiotics negatively affect the growth, disease resistance and behaviour in fishes and simultaneous changes in the antioxidant defense system are used as biomarkers.

Chapter 10

ETHANOL INDUCED HISTOPATHOLOGICAL CHANGES IN THE DIFFERENT TISSUES OF *OREOCHROMIS MOSSAMBICUS* (PETERS)

Contents

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10.2B Materials and Methods
10.2B.1 Preparation of tissue samples for histopathological studies.
10.2B.2 Major steps involved in histological procedures.
10.3C Results
10.4D Discussion

10.1A. INTRODUCTION

Pollution of aquatic environment by industrial effluents has been a major concern in recent years. The ability of such pollutants to disrupt the normal functioning of the aquatic ecosystem depends mainly upon the concentration of the contaminants contained in them. It also depends upon the physico-chemical characteristics of the waste water effluent. With the rapid rise in the price of crude oil and projected decrease in oil supplies, alternative fuels have started receiving considerable attention (Hill *et al.*, 2006). Ethanol has been gaining momentum as a viable alternative fuel. In addition to its common pharmaceutical and beverage use, ethanol is being used as a fuel additive, gasoline enhancer and as an alternative fuel. The production of ethanol from cellulosic materials by direct bioconversion is highly encouraging and its commercial production has already been established in countries like Brazil, Canada and USA. As a result, production of ethanol from renewable carbohydrate materials for use as an alternative liquid fuel has been attracting a worldwide interest. For every litre of ethanol being produced, about 15 litres of effluent is generated which contributes to high organic pollution load in the

receiving water bodies (Singh and Nigam, 1995). This in turn brings about changes in the biochemistry, physiology and histology of the aquatic biota (Ciesarova *et al.*, 1996). There has been a report by Sahai *et al.* (1979) stating that waste effluents generated after fermentation when released into the environment without proper treatment causes environmental pollution. Kumar and Ramkumar (1997) findings also reiterate the pollution of aquatic environment by domestic waste as well as from untreated or partially treated industrial effluents which in turn contributes to massive mortality of fish as well as other important aquatic biota. Although toxicant impairs the metabolic and physiological activities of the organisms, physiological studies alone do not provide a complete understanding of the tissue damage under toxic stress. Hence it is useful to have an insight into histological analysis. The extent of severity of tissue damage is a consequence of the concentration of toxicant and it is also time dependent. Fish is widely used in toxicologic pathology as suitable model to evaluate the health of aquatic ecosystems (Law, 2003). Singh *et al.* (1990) studied the environmental pollution caused by heavy metals and its effects on certain fresh water fishes. Histopathological examination has been increasingly recognized as a valuable tool for the assessment of the impact of environmental pollutants on fishes (Heath, 1995; Teh *et al.*, 1997). Fish gill has been referred to as a multifunctional organ responsible for respiration, osmoregulation, acid-base balance and nitrogenous waste excretion. Under natural conditions, they are the first target of water-borne pollutants, as they form the primary route of uptake of water and associated substances and have large surface areas which are in direct contact with noxious substances (Tkatcheva *et al.*, 2004). Liver is recognized as the center for metabolism and detoxification in piscine body. They are the primary organs required for biotransformation of organic xenobiotics. The teleost kidney plays a principal role in the accumulation, detoxification, and excretion of toxins (Eisler, 1998; WHO, 1991). The heart in teleosts which is situated within the pericardium, anterior to the main body cavity and ventral to the pharynx comprises of four chambers which play a very important role in circulation of blood. Histopathological studies have been conducted to establish the relationships between contaminant exposure and various biological responses. Hence the present study was carried out to assess the histological changes brought about in the vital tissues such as kidney, liver, gills, and heart of *O. mossambicus* when subjected to different sub lethal concentrations of ethanol for 21 days with a periodical sampling at 7 days of exposure.

10.2B MATERIALS AND METHODS

Collection, maintenance, acclimatization of fish, determination of LC_{50}, bioassay method and experimental design for ethanol based study was the same as that described in chapter 1, section 1.2B.1 to 1.2B.5.

10.2B.1 Preparation of tissue samples for histopathological studies

O. mossambicus was exposed for 21 days to sub lethal concentrations of ethanol (0.65 g/l, 1.3 g/l and 2.6 g/l) in the laboratory conditions. After 7 and 21 days of exposure, the fishes were killed by ordinary pithing (by damaging the brain) using a sharp needle. Tissues such as gills, liver, heart and kidney were removed from control and experimental fish. They were then rinsed in distilled water to remove blood and other body fluids. The isolated tissues were then immediately fixed in 10% neutral buffered formalin for 24 hours.

10.2B.2 Major steps involved in histological procedures

Histopathological analysis involves major steps such as fixation, tissue processing, decalcification, section cutting and staining (Raphael, 1976).

a. Fixation

Fixation is the process of preserving, hardening and preventing postmortem changes in the tissues.

Reagents

10% Neutral buffered formaldehyde solution pH 7.0. To 100 ml of 37-40% formaldehyde solution, 900 ml of Dist H_2O, 4 g of sodium phosphate monobasic and 6.5 g of sodium phosphate dibasic were added. pH was adjusted to 7.

Procedure

Tissues were placed in fixative immediately after removal from the body. Tissue blocks were then cut to thickness of about 5 mm so that the fixative could readily penetrate throughout the tissue in a reasonably short time. The volume of

fixative employed was 15-20 times that of the tissue to be fixed. The duration of fixation was 24 hrs. They were then washed in running water overnight and was then stored in 70% alcohol.

b. Tissue Processing

This step involves dehydration, clearing and infiltration of the tissue with paraffin. Dehydration using 50-70% dilution of alcohol prevents distortion that would occur to the tissues. Clearing helps in bringing about miscibility between alcohol and paraffin. The tissue was then impregnated and embedded with molten paraffin.

Reagents

Ethyl alcohol, Xylene, Paraffin

Procedure

The following time schedule was used to make paraffin wax blocks for histological studies.

1. Tissues were washed overnight in running water.
2. A sudden change of the tissues from aqueous medium to alcohol concentrations of 30%, 50% and70% was carried out.
3. The tissues were stored in fresh 70% alcohol. At this stage, the tissue can be stored until further processing.
4. Tissues were then dehydrated by transferring them sequentially to 70%, 80%, 90%, 95% alcohol for 1 hour each.
5. The tissues were subjected to 2 changes for 1 hour each in 95% alcohol.
6. They were transferred to absolute alcohol, where two changes for 1 hour each was done.
7. The tissues were placed in 1:1 mixture of absolute alcohol and xylene (xylol) for 1 hour.
8. Tissues were then placed in acetone for complete dehydration for 1 hour.

9. Tissues were cleared in xylene. This step was carried out until the tissues become translucent.
10. Tissues were left overnight in a mixture of xylene and paraffin.
11. The tissues were in filtered in molten paraffin wax of melting point 60 – 62^0 C, with 2-3 changes.

The in filtered block was then embedded in molten paraffin wax. During the process of embedding, the tissue blocks were oriented in such a way that sections could be cut in the desired plane of the tissue. The block, after cooling was trimmed to a suitable size and was fixed on a metal object holder. The block was further trimmed so that paraffin overlying the piece of tissue was excluded and an adequate area of the tissue facing the knife was exposed.

c. Decalcification

Principle

Decalcification is the term applied to organic tissues which have been infiltrated with calcium salts. These salts were removed to assure that the specimen is soft enough to allow section cutting.

Reagents

1. 10% EDTA (dipotassium salt)

Procedure

Gill tissues were cut into small pieces with fine saw. After sufficient fixation, pieces were placed in a large amount of decalcifying solution containing 10% EDTA. Stirring and heating hastens decalcification. They were then suspended in the upper $1/3^{rd}$ of fluid during decalcification, so that calcium salts sink to the bottom of the container. Since the decalcifying solution contains acid, the gill tissues were washed thoroughly to remove acid before subsequent processing.

It is very important to determine the end point to remove the tissues from the decalcifying fluid once the decalcification is accomplished. If this is not done, the chances of subsequent good staining reactions are reduced by 10% for every 2 hrs as

long as the tissue remains in the decalcifying fluid. For this approximately 5 ml of decalcifying fluid (from the bottom of container) which has been in contact with the tissue for 6-12 hr was drawn. 5 ml each of 5% ammonium hydroxide and ammonium oxalate were added. It was mixed and was let to stand decalcified. The decalcifying solution was changed and the test was performed at a later time. When a milky solution is no longer obtained from such a mixture, the specimen is said to be completely decalcified.

d. Section cutting

Sections were cut at 5μ thickness and were floated in a water bath between 38-49^{0}C. The sections from the water were then mounted on clean glass slides smeared with Mayer's egg albumin. They were then dried on a hot plate at about 50^{0}C for 30 min. The sections on the slides were kept ready for staining.

e. Staining procedure using Haematoxylin and Eosin (Luna, 1968)

Reagents

1. Mayer's Haematoxylin stain: Dissolved 50 g of ammonium or potassium alum in 1L of water without heating. To this 1g of haematoxylin was added. Then 0.2 g sodium iodate, 1g citric acid and 50 g of chloral hydrate were added. It was then shaken well until all the components got completely dissolved in solution. The final colour of the stain obtained was reddish violet which can be stored for a month.
2. Stock eosin solution (1%): Dissolve 1 g of Eosin Y (water soluble) in 20 ml distilled water. This was made up to 100 ml with 95% alcohol.
3. Working eosin solution: Diluted 1 part of the stock eosin solution with 3 parts of 80% alcohol. 0.5 ml of glacial acetic acid was added for every 100 ml of stain.

Procedure:

The slides containing the section were processed serially as follows:

1. The slides were transferred to xylene: absolute alcohol (1:1) (xylol) and were subjected to two changes for 5 min each.

2. They were hydrated by passing through a descending series (95%, 90%, 80%, 70%, 50% and 30%) of alcohol for 5 minute each.
3. The slides were washed in running tap water for 5 minutes.
4. They were stained using haematoxylin for 10 minutes.
5. The stained slides were washed in running tap water for 10 minutes.
6. The slides were counter stained by keeping in Eosin working solution ranging from 15 sec to 5 minutes.
7. The stained slides were dehydrated by passing them through an ascending series (30%, 50%, 70%, 80%, 90% and 95%) of alcohol for 3 minutes each.
8. They were subjected to 2 to 3 dips of 95% alcohol in which two changes were provided.
9. They were followed by 100% alcohol. Two changes were provided for 1 to 2 minutes each.
10. The slides were then placed in acetone. Two changes were provided for 3 minutes each.
11. The slides were dipped in xylene: absolute alcohol (1:1). Two changes were provided for 3 minutes each.
12. Finally the slides after clearing with xylene (2 changes) were mounted in DPX medium. They were examined under microscope (Leica DM/LS Type 020-518.500501095) with camera attachment (Leica) and were photographed at both high as well as low power resolutions. The nuclei stained blue and cytoplasm in various shades of pink.

10.3C RESULTS

Plate 10.1 to 10.4 depict the normal and altered histology of tissues such as gill, liver, heart and kidney of *O. mossambicus* exposed to various sub-lethal concentrations (0.65 g/l, 1.3 g/l and 2.6 g/l) of ethanol for 7 days and 21 days.

* Plate. 10.1a and b depict the normal gill architecture of control fishes with intact primary and secondary lamellae. On exposure to different concentrations of ethanol *O. mossambicus* exhibited marked changes.

* Changes observed in the gill of *O. mossambicus* during 7 days of exposure to different concentrations of ethanol

At 0.65 g/l ethanol, epithelial hyperplasia, dilated blood vessel as well as oedema were observed (Plate 10.1b). At 1.3 g/l, haemorrhage and telangiectasis were observed (Plate 10.1c). At 2.6 g/l, gill aneurysms followed by sloughing were observed towards the base of the primary gill filament and at the edges of the secondary gill lamellae (Plate 10.1d).

* Changes observed in the gill of *O. mossambicus* during 21 days of exposure to different concentrations of ethanol

At 0.65 g/l ethanol, haemorrhages, hyperplasia and clubbing were seen (Plate 10.1e). At 1.3 g/l ethanol, necrosis of the gills and haemorrhages (Plate 10.1f) were found. At 2.6 g/l ethanol, complete epithelial desquamation and haemorrhages (Plate 10.1g) were observed.

* Changes observed in the liver of *O. mossambicus* during 7 days of exposure to different concentrations of ethanol

No histopathological changes were observed in the liver of control fish. They exhibited normal liver architecture with hepatocytes (Plate 10.2a). When they were exposed to different concentrations of ethanol liver sections showed marked structural changes. At 0.65 g/l ceroid pigmentation, focal area of necrosis, more stainable cytoplasm (Plate 10.2b) followed by marked fatty changes (Plate 10.2c) were observed. At 1.3 g/l elongated biliary proliferation (Plate 10.2d) was observed. At 2.6 g/l hepatic cord disruption, pyknotic nuclei, extensive proliferation of the biliary epithelium and ceroid pigmentation (Plate 10.2e) were noted.

* Changes observed in the liver of *O. mossambicus* during 21 days of exposure to different concentrations of ethanol

At 0.65 g/l connective tissue proliferation (Plate 10.2f) was noted. At 1.3 g/l loss of parenchymatous structure as well as hepatocyte necrosis (Plate 10.2g) was noted. At 2.6 g/l complete disruption of hepatic cords, pancreatic tissue necrosis,

hepatocytic necrosis as well as proliferation of bile duct tubules (Plate 10.2h) were observed.

* Changes observed in the heart of *O. mossambicus* during 7 days of exposure to different concentrations of ethanol

Heart tissue of the control fish exhibited normal architecture (Plate 10.3a). When being subjected to 0.65 g/l dosage of ethanol, loss of striation, vacuolation of sarcoplasm as well as phagocytic accumulation were noted (Plate 10.3b). At 1.3 g/l loss of muscle fibres, loss of striations, more extensive necrosis, as well as vacuolation of sarcoplasm were observed (Plate 10.3c). At 2.6 g/l hyalinization and loss of striations were observed (Plate 10.3d).

* Changes observed in the heart of *O. mossambicus* during 21 days of exposure to different concentrations of ethanol

On exposure to 21 days at 0.65 g/l ethanol, fragmentation of muscle fibres and accumulation of leucocytes towards the periphery (Plate 10.3e) were observed. At 1.3 g/l destruction of muscle fibres and leucocyte accumulation (Plate 10.3f) were observed. At 2.6 g/l fragmentation and necrosis (Plate 10.3f) were observed.

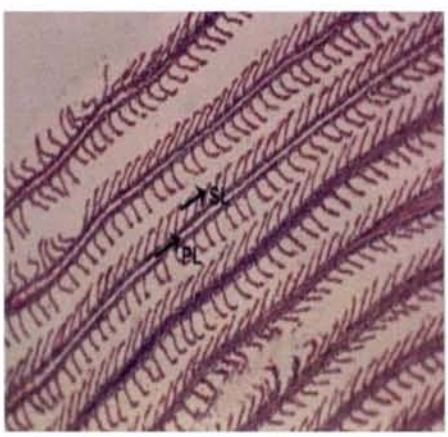
* Changes observed in the kidney of *O. mossambicus* during 7 days of exposure to different concentrations of ethanol

On exposure to 7 days in the case of control fish, kidney of *O. mossambicus* comprised of very well developed glomeruli and a system of tubules (Plate 10.4a). At 0.65 g/l vacuolation of epithelial cells, thickening of Bowman's capsule, shrinkage of glomeruli and adhesion were observed (Plate 10.4b). At 1.3 g/l shrinkage of glomeruli, necrosis of epithelial cells, loss of tubules and condensed nucleus were observed (Plate 10.4c). At 2.6 g/l adhesion, thickening of the Bowman's capsule (Plate 10.4d) and intercapillary thickening were observed (Plate 10.4e).

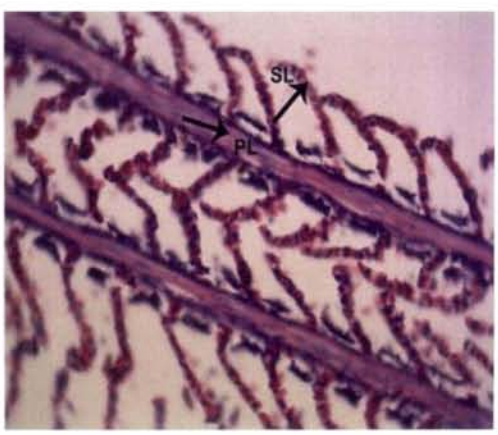
* Changes observed in the kidney of *O. mossambicus* during 21 days of exposure to different concentrations of ethanol

On exposure to 21 days at 0.65 g/l adhesion, necrosis as well as fibrosed area were observed (Plate 10.4d). At 1.3 g/l thickening of Bowman's capsule, intercapillary wall thickening, shrinkage of glomeruli was observed (Plate 10.4e). At 2.6 g/l glomerular thickening, tubular necrosis, glomerular necrosis, thickening of bowman's capsule (Plate 10.4f) were noted.

Plate10.1 Histopathological changes observed in the gill tissues of *O. mossambicus* exposed to different concentrations of ethanol for 7 days and 21 days.



(a)



(b)

Plate 10.1a : Photomicrograph of the control gill of *O. mossambicus* showing normal gill architecture with primary gill lamellae (PL) and secondary gill lamellae (SL). (H & E × 10)

Plate 10.1a: Photomicrograph of the control gill of *O. mossambicus* showing normal gill architecture with primary gill lamellae (PL) and secondary gill lamellae (SL). (H & E × 40)

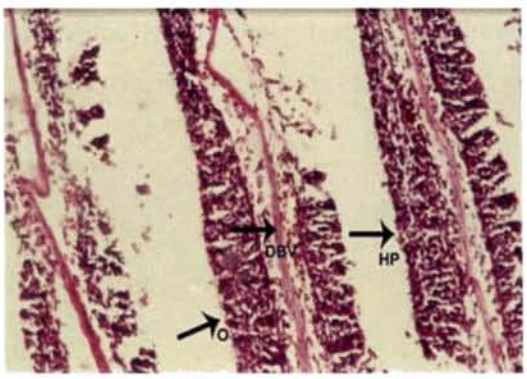


Plate10.1b: Photomicrograph of the gill of *O. mossambicus* exposed for 7 days at 0.65g/l ethanol showing hyperplasia of the epithelium (HP), dilation of the blood vessel (DBV) and oedema (O). (H & E × 20)

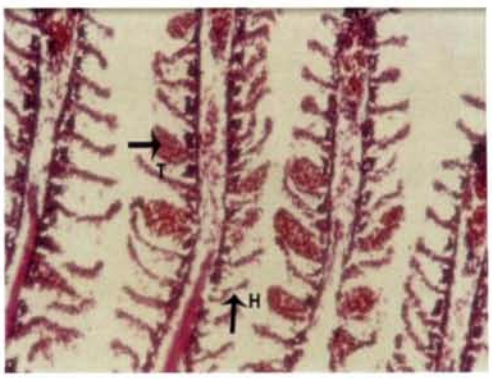


Plate10.1c: Photomicrograph of the gill of *O. mossambicus* exposed for 7 days at 1.3g/l ethanol showing haemorrhages (H) and telangiectasis (T). (H & E × 20)

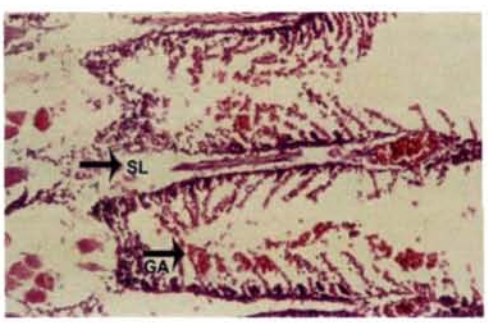


Plate10.1d: Photomicrograph of the gill of *O. mossambicus* exposed for 7 days at 2.6g/l ethanol showing gill aneurysm (GA), sloughing towards the base of the primary gill filament (SL) as well as at the edges of the secondary gill lamellae. (H & E × 20)

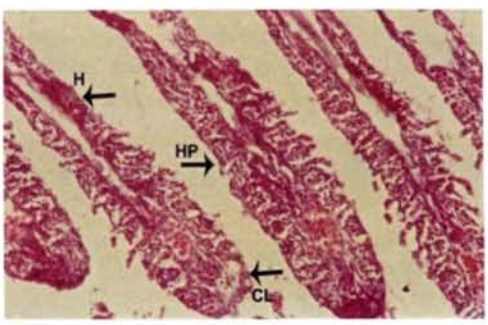


Plate10.1e: Photomicrograph of the gill of *O. mossambicus* exposed for 21 days at 0.65g/l ethanol showing haemorrhages (H), hyperplasia (HP) and clubbing (CL). (H & E × 20)

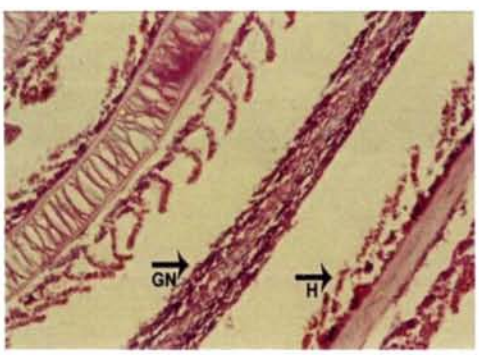


Plate10.1f: Photomicrograph of the gill of *O. mossambicus* exposed for 21 days at 1.3g/l ethanol showing gill necrosis (GN) and haemorrhages (H). (H & E × 20)

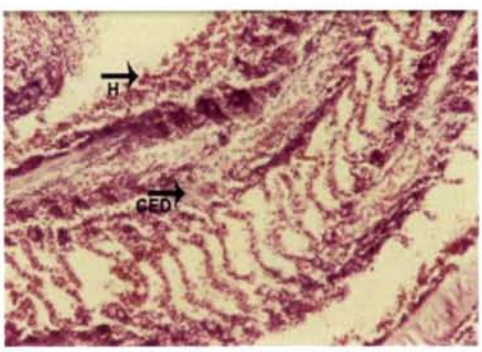


Plate10.1g: Photomicrograph of the gill of *O. mossambicus* exposed for 21 days at 2.6g/l ethanol showing complete epithelium desquamation (CED) as well as haemorrhages (H). (H & E × 20)

Plate 10.2 Histopathological changes observed in the liver tissues of *O. mossambicus* exposed to different concentrations of ethanol for 7 days and 21 days.

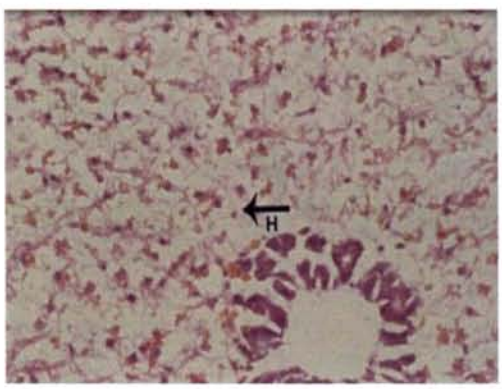


Plate10.2a: Photomicrograph of the control liver of *O. mossambicus* showing normal liver structure with hepatocytes (H). (H & E × 40)

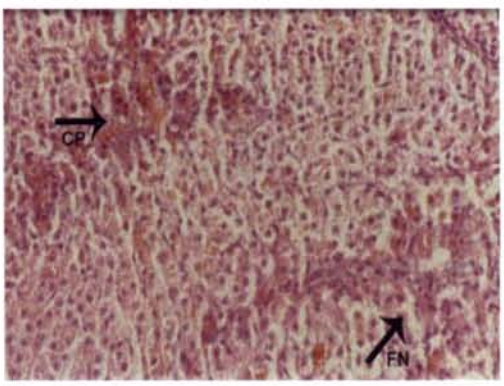


Plate10.2b: Photomicrograph of the liver of *O. mossambicus* exposed for 7 days at 0.65g/l ethanol showing ceroid pigmentation (CP) as well as focal area of necrosis (FN). (H & E × 40)

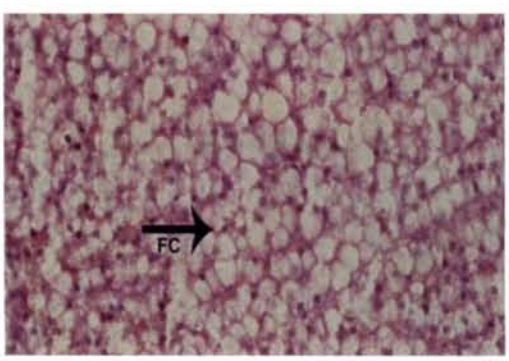


Plate10.2c: Photomicrograph of the liver of *O. mossambicus* exposed for 7 days at 0.65g/l ethanol showing fatty changes (FC). (H & E × 40)

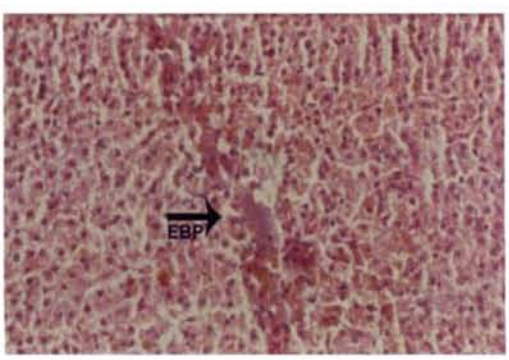


Plate10.2d: Photomicrograph of the liver of *O. mossambicus* exposed for 7 days at 1.3g/l ethanol showing elongated biliary proliferation (EBP). (H & E × 40)

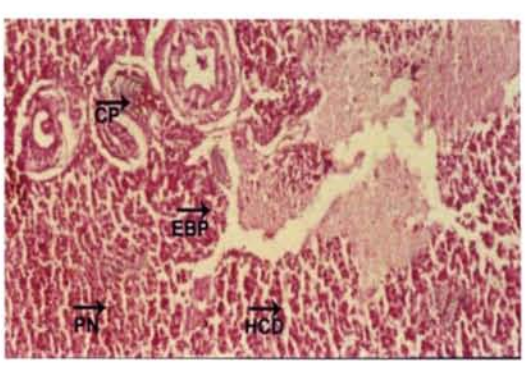


Plate10.2e: Photomicrograph of the liver of *O. mossambicus* exposed for 7 days at 2.6g/l ethanol showing hepatic cord disruption (HCD), pyknotic nuclei (PN), extensive proliferation of the biliary epithelium (EPBE) and ceroid pigmentation (CP). (H & E × 40)

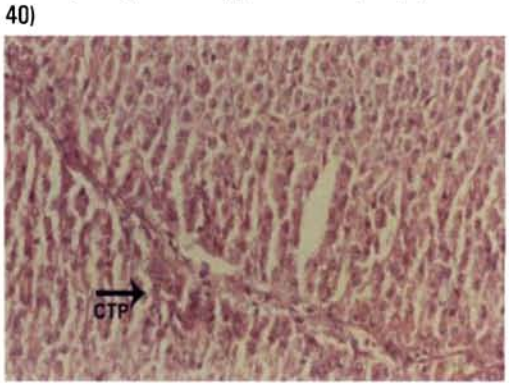


Plate10.2f: Photomicrograph of the liver of *O. mossambicus* exposed for 21 days at 0.65g/l ethanol showing connective tissue proliferation (CTP). (H & E × 40)

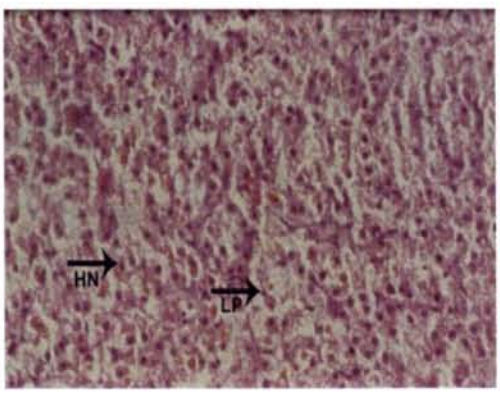


Plate10.2g: Photomicrograph of the liver of *O. mossambicus* exposed for 21 days at 1.3g/l ethanol showing loss of parenchymatous structure (LP) and hepatocyte necrosis (HN). (H & E × 40)

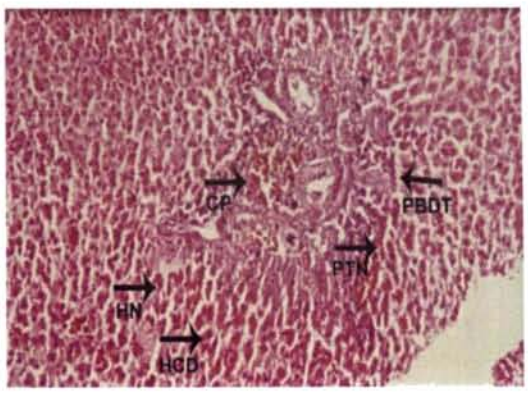


Plate10.2h: Photomicrograph of the liver of *O. mossambicus* exposed for 21 days at 2.6g/l ethanol showing hepatic cord disruption (HCD), ceroid pigmentation (CP), pancreatic tissue necrosis (PTN), hepatocyte necrosis (HN) and proliferation of bile duct tubules (PBDT). (H & E × 40)

Plate 10.3 Histopathological changes observed in the heart tissues of *O. mossambicus* exposed to different concentrations of ethanol for 7 days and 21 days.

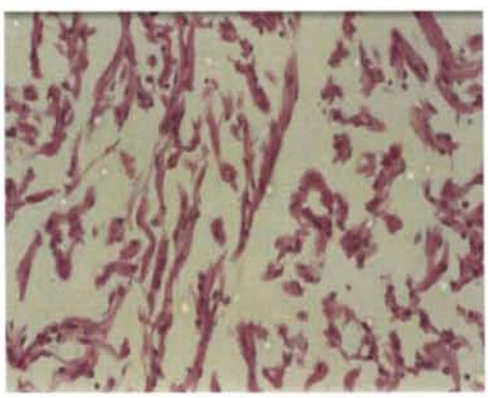


Plate10.3a: Photomicrograph of the control heart o *O. mossambicus* showing normal architecture. (H & E × 40)

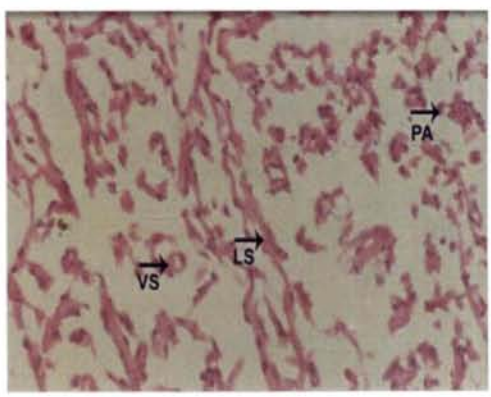


Plate10.3b: Photomicrograph of the heart of *O. mossambicus* exposed for 7 days at 0.65g/l ethanol showing loss of striation (LS), vacuolation of sarcoplasm (VS) and phagocyte accumulation (PA). (H & E × 40)

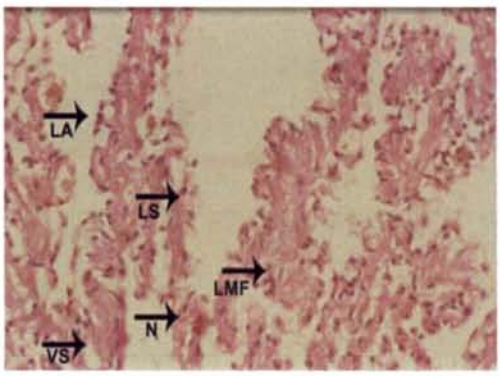


Plate10.3c: Photomicrograph of the heart of *O. mossambicus* exposed for 7 days at 1.3g/l ethanol showing loss of muscle fibres (LMF), loss of striations (LS), extensive necrosis (N) and vacuolation of sarcoplasm (VS). (H & E × 40)

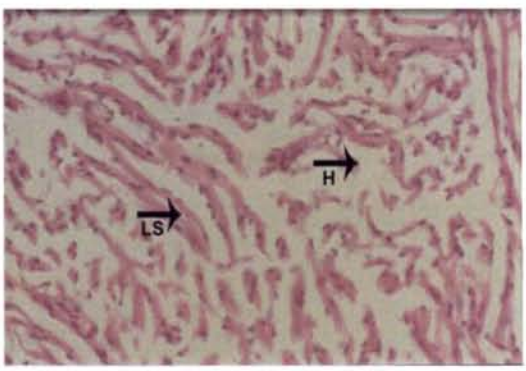


Plate10.3d: Photomicrograph of the heart of *O. mossambicus* exposed for 7 days at 2.6g/l ethanol showing hyalinization (H) and loss of striations (LS). (H & E × 40)

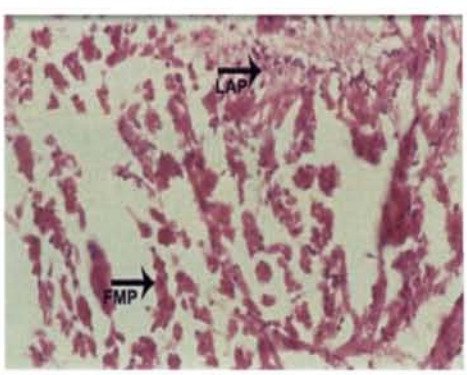


Plate10.3e: Photomicrograph of the heart of *O. mossambicus* exposed for 21 days at 0.65g/l ethanol showing fragmentation of muscle fibres (FMF) and leucocyte accumulation at the periphery (LAP). (H & E × 20)

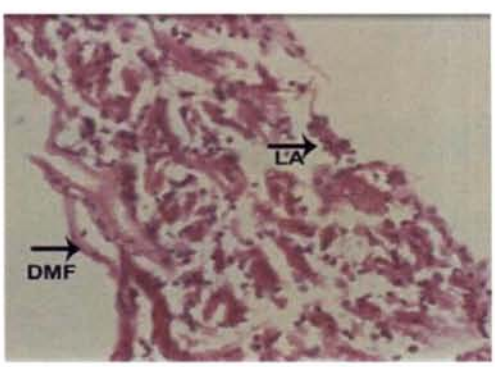


Plate10.3f: Photomicrograph of the heart of *O. mossambicus* exposed for 21 days at 1.3g/l ethanol showing destruction of muscle fibres (DMF) and leucocyte accumulation (LA). (H & E × 20)

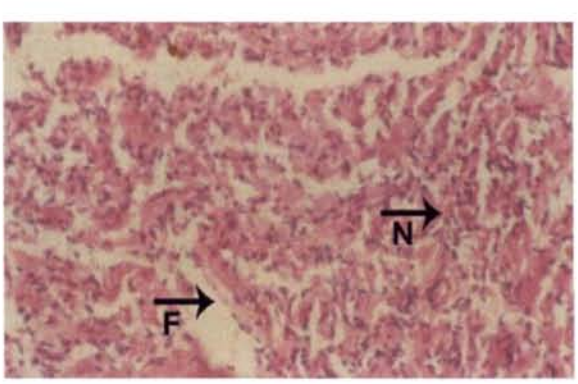


Plate10.3g: Photomicrograph of the heart of *O. mossambicus* exposed for 21 days at 2.6 g/l ethanol showing fragmentation (F) and necrosis (N). (H & E × 20)

Plate 10.4 Histopathological changes observed in the kidney tissues of *O. mossambicus* exposed to different concentrations of ethanol for 7 days and 21 days.

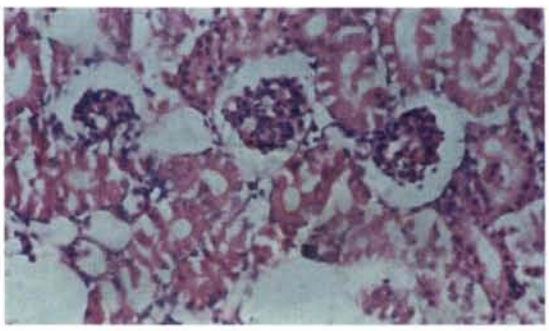


Plate 10.4a: Photomicrograph of the control kidney of *O. mossambicus* showing normal architecture. (H & E × 40)

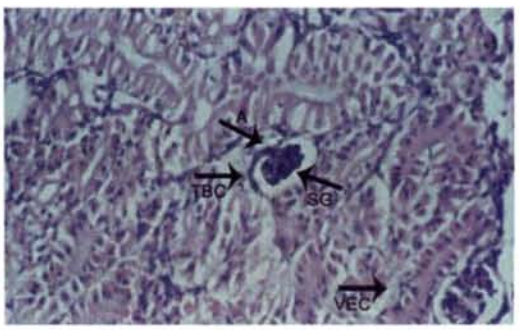


Plate10.4b : Photomicrograph of the kidney of *O. mossambicus* exposed for 7 days at 0.65g/l ethanol showing vacuolation of epithelial cells (VEC), thickening of bowman's capsule (TBC), shrinkage of glomeruli (SG) and adhesion (A). (H & E × 40)

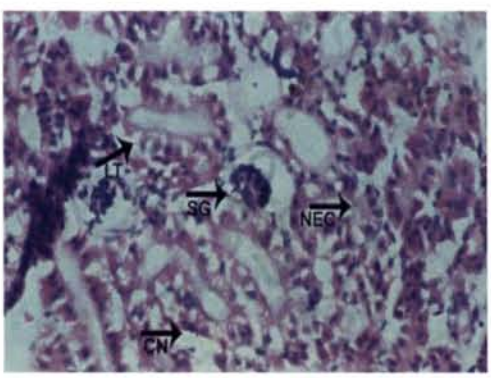


Plate10.4c: Photomicrograph of the kidney of *O. mossambicus* exposed for 7 days at 1.3g/l ethanol showing shrinkage of glomeruli (SG), necrosis of epithelial cells (NEC), loss of tubules (LT) and condensed nucleus (CN). (H & E × 40)

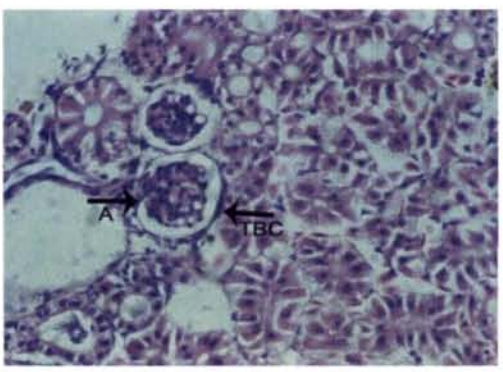


Plate10.4d: Photomicrograph of the kidney of *O. mossambicus* exposed for 7 days at 2.6g/l ethanol showing adhesion (A) and thickening of the bowman's capsule (TBC). (H & E × 40)

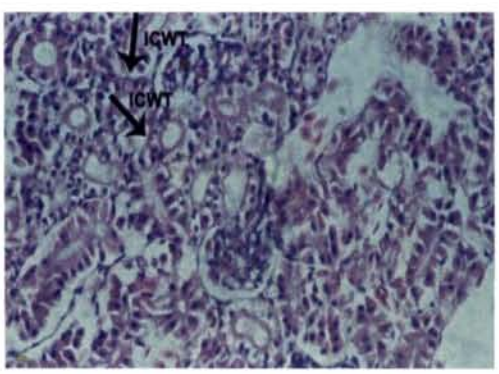


Plate10.4e: Photomicrograph of the kidney of *O. mossambicus* exposed for 7 days at 2.6g/l ethanol showing intercapillary thickening (ICWT). (H & E × 40)

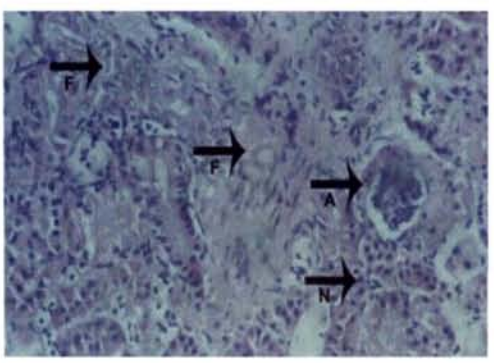


Plate10.4f: Photomicrograph of the kidney of *O. mossambicus* exposed for 21 days at 0.65g/l ethanol showing adhesion (A), necrosis (N) and fibrosed area (F). (H & E × 40)

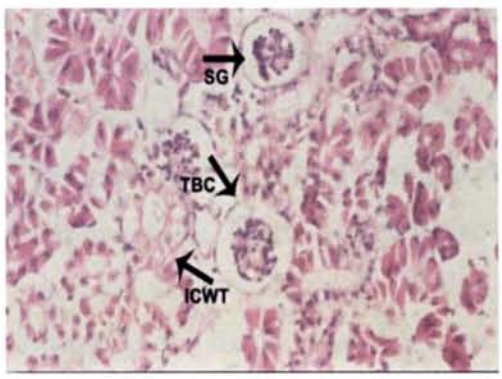


Plate10.4g: Photomicrograph of the kidney of *O. mossambicus* exposed for 21 days at 1.3g/l ethanol showing thickening of bowman's capsule (TBC), intercapillary wall thickening (IWT) and shrinkage of glomeruli (SG). (H & E × 40)

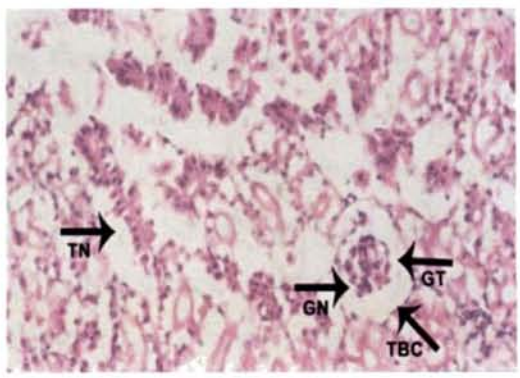


Plate10.4h: Photomicrograph of the kidney of *O. mossambicus* exposed for 21 days at 2.6g/l ethanol showing glomerular thickening (GT), tubular necrosis (TN), glomerular necrosis (GN) and thickening of bowman's capsule (TBC). (H & E × 40)

10.4D DISCUSSION

Extensive studies have been carried out on histopathology of liver, kidney, gills and heart of fresh water fishes being exposed to pollutants (Athikesavan *et al.*, 2006; Anithakumari and Sreeramkumar, 1997; Rana and Sudhir, 1999; Raghu Prasad and Belazutshi, 2007; Tilak *et al.*, 2001; Venkataramana *et al.*, 2001). But in the case of *O. mossambicus*, histopathology of different tissues when exposed to ethanol has not been reported so far. Hence this study can be considered as a pioneer attempt. According to Vander Oost *et al.* (2003) fishes are generally considered as the most feasible organisms employed for pollutant monitoring in aquatic systems. Fish being located at the top of the aquatic food chain, seems to be a highly visible resource. They are in direct contact with pollutants in the water via their gills and their body surface. According to Johnson *et al.* (1993) histopathology serves as a rapid method to detect the effects of irritants in various organs. The exposure of fish to chemical contaminants induces a number of lesions in different organs (Bucke *et al.*, 1996). The gill morphology of the control tilapia is very much similar to that of other teleost fish species as stated by Wilson and Laurent (2002). Fish gills are vulnerable to pollutants in water because of their large surface area and external location. For this reason, they are considered to be the most appropriate indicators of

water pollution levels (Alazemi *et al.*, 1996). According to Timbrell (1991), toxic compounds have the potential to cause maximum harm to the tissues and organs that come into contact with them first. The histological examination revealed several structural and functional changes in the gills. The gills, being delicate structures, get affected easily when the surrounding media is contaminated (Roy and Munshi, 1991). In the case of aquatic organisms, gills are considered to be vital organs that play an important role in the transport of respiratory gases and regulation of the osmotic and ionic balance. Toxic substances may cause damage to gill tissues, thereby reducing the oxygen consumption and disrupting the osmoregulartoy function of aquatic organisms (Ghate and Mulherkar, 1979). Healthy and control gill is characterized by the presence of primary lamellae along with the secondary lamellae confirming to the general architectural design of the tissue. In the present study no histopathological changes were observed in the gills of the control fish during laboratory rearing. Hyperplasia of epithelial cells observed in the present study serves as physical and physiological defense mechanism against the toxicant ethanol. It also increases the diffusion distance thus affecting the exchange of gases. This theory has been supported by the findings of Mallatt (1985). Nowak (1992) found similar histological changes in the gills of fishes when intoxicated by residues of endosulfan. Oedema cited in this study is referred to as a defense mechanism that reduces the branchial superficial area of the fish which comes in contact with the external milieu. These mechanisms also increase the diffusion barrier to the pollutant. The same findings have been noted by Lauren and McDonald (1985). Van Heerden *et al.* (2004) also observed oedema in the gills of rainbow trout *Oncorhynchus mykiss* when exposed to copper. Pandey *et al.* (1996) also observed oedema followed by hyperplasia in the gills of an estuarine mullet, *Liza parsia* when exposed to mercuric chloride for 7 days. Dilation of the blood vessels observed in the present study is due to increased permeability induced by the immediate exposure to ethanol. This helps in the free passage of ethanol into the blood stream. Balah *et al.* (1993) found similar changes in the gills of *O. niloticus* when exposed to heavy metals. Telangiectasis observed in this study explains the state of asphyxia of the fish when subjected to ethanol toxicity. Telangiectatic secondary lamellae was seen in the findings of Maleeya *et al.* (2008) in the gill of *O. niloticus* when

subjected to acute alachlor exposure. Similar observations were cited by Llyod (1965), who too observed sloughing of the cells in the gills of rainbow trout when exposed to zinc, lead and copper. Gill aneurysm indicates impaired respiratory function. This is related to the rupture of the pillar cells brought about by the extensive flow of blood. It also arises due to the direct effects of contaminants on the pillar cells. The same inference has been cited by Poleksic and Mitrovic-Tutundzic (1994); Heath (1987) and Martinez *et al.* (2004). Observations made by Maleeya *et al.* (2008) also supported this. Haemorrhages, hyperplasia and clubbing reduces the respiratory area thereby reducing the respiratory and osmoregulatory potential of the fish *O. mossambicus*. It also indicates a decrease in energy metabolism due to the degeneration of respiratory epithelium and the damage brought to the gill tissue which may finally result in tissue hypoxia. These pathological changes observed in respiratory organs might have resulted in a shift from aerobic to anaerobic pathway of metabolism in the tissues of fish under pollutant stress. The clubbed appearance of lamellae refers to lamellar hyperplasia from where cells are derived from primary lamellae and migrate towards the distal end. This finally results in accumulation of cells at the leading edge of secondary lamella, which colloquially is termed as 'clubbing' of lamellae. The findings of Chhaya *et al.* (2007) supported the above statement. Tilak *et al.* (2001) also observed similar changes in the gills of *Ctenopharyngodon idella* (Valenciennes) when exposed to technical fenvalerate. Athikesavan *et al.* (2006) also obtained similar results in the gill of *Hypophthalmichthys molitrix* (Valenciennes) when subjected to nickel toxicity. Cengiz (2006) also observed similar histopathological effects in the gills of common carp when subjected to deltamethrin. Ilhan and Erol (2007) cited similar changes in the gills of rainbow trout when exposed to sub lethal concentrations of endosulfan. Velmurugan *et al.* (2007) observed complete epithelium desquamation as well as haemorrhages in the gill tissues of *Cirrhinus mrigala* when treated with monocrotophos. Rao *et al.* (2006) also reported similar pathology in fish exposed to sub lethal concentration of profenofos. Gill alterations such as epithelial hyperplasia observed are usually directly related to gill function disorders, which may affect the physiology or cause the death of fish (Smart, 1976). Necrosis of the gills, haemorrhages followed by complete epithelium desquamation have been considered

as a common reaction towards strong water pollution arising due to the presence of ethanol. Daye and Garside (1976) also cited similar histopathological changes in the gills of brook trout, *Salvelinus fontinalis* (Mitchill), when exposed to acute and chronic level of pH. Haemorrhages can be interpreted as a reflection of the direct action of ethanol on the tissue. The findings of Temmink *et al.* (1983) supported the above statement.

In the case of teleosts, the liver is a bilobed gland comprising of two tissue compartments, the parenchyma (comprising of hepatocyte) and stroma (comprising of hepatopancreas, bileduct, blood vessels and connective tissue). The parenchymatous cells forming hepatic cords lie irregularly and get separated by blood sinusoids. Hepatocytes are polygonal cells with a prominent central spherical nucleus and a densely stained nucleolus. Each sinusoid consists of an outer peripheral connective tissue and an inner lining of endothelial cells. Stentiford *et al.* (2003) utilized liver histopathology as an indicator of environmental stress since it provides a definite biological end-point of historical exposure). Liver not only acts as a storage organ, but is also considered to be the primary site for detoxification mechanisms (Wannee *et al.*, 2006). It is said to be to be the primary organ employed for detoxification of xenobiotics (Metelev *et al.*, 1971). Liver is considered to be the primary site of alcohol metabolism. As alcohol is broken down in the liver, a number of potentially dangerous by-products are generated such as acetaldehyde and highly reactive molecules called as free radicals perhaps more so than alcohol itself. It is in fact these products that contribute to alcohol – induced liver damage. In the case of control fishes, the liver exhibited a normal architecture with hepatocytes presenting a homogenous cytoplasm, and a large central or sub central spherical nucleus with no pathological abnormalities. *O. mossambicus* when exposed to different sub lethal concentrations of ethanol for 21 days followed by a periodical sampling at 7 days exhibited marked pathological changes. These changes were referred to as by-products of catabolism. This explains the stress seen in *O. mossambicus* when subjected to ethanol toxicity. Ceroid pigmentation is mainly brought about by the free radical damage occurring to the hepatocytes. Similar finding was suggested by Mahjoor and Lohasian (2008) in the liver of rainbow trout when subjected to chlorine toxicity. Focal area of necrosis observed in the present study on *O.*

mossambicus has resulted from the excessive work brought about by the fish to get rid of the toxicant from its body during the process of detoxification. It might have also developed by the inability of the fish to regenerate new liver cells. The findings of Ayoola (2008) supported this. Necrotic changes were also observed in the liver of Nile tilapia *(O. niloticus)* when subjected to glyphosate herbicide toxicity. Fatty changes are the characteristic feature of liver damage. The changes in fat content may be due to the increased mobilization and transport of fat to the liver. James Kang *et al.* (2002) who observed accumulation of fat on acute exposure when treated with ethanol in the hepatocytes of the wild type mice supports the present finding. Mild to moderate hepatocellular fatty changes have been reported with 95% ethanol in the case of a few birds when exposed for 7 days by Allen *et al.* (1981). Purohit *et al.* (2004) have mentioned that, in the initial stages of the alcoholic liver disease fat accumulation found in hepatocytes leads to the development of fatty liver (steatosis), which may progress to hepatitis and fibrosis, and finally to liver cirrhosis. Rouiller (1964) stated that fatty changes are the characteristic feature of liver damage. Elongated biliary proliferation was cited by Udita *et al.* (2004) in male and female rats when treated with cadmium. Marked histopathological changes such as hepatic cord disruption, pyknotic nuclei, extensive proliferation of the biliary epithelium and ceroid pigmentation were supported by Tilak *et al.* (2001) who observed similar changes in the liver of *Ctenopharyngodon idella* (Valenciennes) when exposed to technical fenvalerate. Loss of parenchymatous structure and hepatocyte necrosis were cited by Serdar *et al.* (2008) in the liver of two fish species *Barbus capito pectoralis* and *Chondrostoma nasus* in the buyuk menderes river in Turkey. Extensive proliferation of the biliary epithelium in the liver of *O. mossambicus* when exposed to ethanol revealed that the metabolic storage products were affected. The findings of Takashima and Hibiya (1995) supported this. Hepatocyte necrosis was brought about by the wide spread inflammation as well as destruction of the liver brought about by the increase concentration of ethanol. This result agreed with the findings of Authman and Abbas (2007) who stated that the liver has an important role in detoxification of endogenous waste products as well as of externally derived toxins. The occurrence of necrosis is said to be one of the most visible damages observed in liver tissues when subjected to ethanol toxicity.

Manahan (1991) had explained necrosis as a consequence of enzymatic inhibition, damages in the cellular membrane integrity, and disturbances in the synthesis of proteins and carbohydrate metabolism. It can also be related with xenobiotic concentration arising during the detoxifying process. Monica *et al.* (2007) have also recorded marked hepatic necrosis with CCl_4 induced toxicity in rats. Histopathological changes such as hepatic cord disruption, pancreatic tissue necrosis, hepatocyte necrosis and proliferation of bile duct tubules were found. This has been brought about by tissue hypoxia. Necrosis found in parenchymatous tissue is brought about by the liquefaction of hepatocytes. Raghu and Bela (2007) have also observed similar changes in the liver of rohu when subjected to environmental stress. The findings of Illhan and Erol (2007) and Serdar *et al.* (2008) supported the present findings.

The functional unit of the teleostan kidney is the nephron. Morphologically, the nephron of *O. mossambicus* consists of glomerulus, proximal and distal tubules and collecting duct. The glomerulus comprises of a cluster of capillaries surrounded by Bowman's capsule. On the inner side of the Bowman's capsule are found epithelial cells. The Bowman's capsule extends to form the convoluted tubules. Kidney histopathology was well characterized by the presence of proximal and distal tubules, glomeruli and interstitial tissue. The kidney is an important organ having not only excretory function but also other functions such as production of the substances that activates a living body, enzymatic reaction, immunization etc (Subir *et al.*, 2008). The kidneys of fish receive the largest proportion of post branchial blood, and therefore renal lesions might be expected to be good indicators of environmental pollution (Ortiz *et al.*, 2003). No marked histopathological changes were observed in the control fishes of *O. mossambicus*. But when exposed to 7 days and 21 days to different sub lethal concentrations of ethanol, marked histopathological changes were found. Histopathological changes such as vacuolation of epithelial cells, thickening of Bowman's capsule, shrinkage of glomeruli and adhesion refers to that of glomerulonephritis. This is supported by the findings of Roanald Roberts (2001). Vacuolation of the epithelial cells has been demonstrated to be a morphological change consistent with potassium depletion that is a feature of the diuretic stage of acute tubular necrosis. Loss of tubules has also

been referred to as a sign of acute tubular necrosis. Organic solvents, drugs, and poisonous fungi have been incriminated as toxic causes of acute tubular necrosis. The above said findings were supported by Hayder *et al.* (2007) who too observed similar changes in the kidneys of rats when subjected to aflatoxin induced toxicity. Adhesion seen between Bowman's capsule and glomeruli was supported by Koponen *et al.* (2001) who found similar changes in the kidney of feral fish obtained from polychlorinated biphenyl contaminated lake. Shrinkage of glomeruli was noted by Velmurugan *et al.* (2007) in the kidney tissues of *Cirrhinus mrigala* when treated with monocrotophos. Apart from glomerular shrinkage, other histopathological changes such as epithelial cells necrosis, loss of tubules and condensed nuclei were observed. Similar findings were stated by Subir *et al.* (2008) in the kidney of ethanol treated rats. Thickening of Bowman's capsule as well as intercapillary thickening was related to the disturbances of the ionic state of the cells caused by the toxicant ethanol. Fathia *et al.* (2005) also found the same changes in the kidneys of mice when treated with dimethoate which supports the present finding. Fibrosed area followed by tubular necrosis occurs as a result of a degenerative process taking place in the tissue. Fibrosed area also refers to the proliferation of fibroblasts occurring in tissues. Glomerular necrosis and tubular necrosis were cited by Ilhan and Erol (2007) in the kidney of rainbow trout when exposed to sub-lethal concentrations of endosulfan for 21 days. Acharya *et al.* (2001) are of the opinion that chronic ethanol intake induced renal tubular necrosis in the kidneys of rats.

O. mossambicus possesses a heart situated ventrally within the pericardium, anterior to the main body cavity and ventral to the pharynx. Like the mammalian heart it is also four chambered. It also comprises of a muscular tissue consisting of a network of fibers. This gets differentiated into three layers, the outermost epicardium, the thick middle myocardium and the inner most endocardium. It also comprises of bulbus arterosus which has got a thick wall consisting of a mixture of elastic tissue and smooth muscle. Contraction and dilation of the heart are performed by the cardiac muscle and any damage to this muscle may result in dysfunction of the heart and incomplete circulation of blood. Hence, in the present study an attempt was made to study the effect of ethanol on the cardiac muscles of fresh water fish *O. mossambicus*. No histopathological damage was found in the heart of the control

fish. The pathological changes observed during immediate and prolonged exposure revealed that they were dependent upon the time and dosage. This was supported by Samreen *et al.* (2006) who also found similar histopathological changes in the heart of quail chicks *Coturnix coturnix japonica* when administrated with different levels of chrome shaving. Loss of striations, leucocyte accumulation followed by necrosis refers to the indications of damage bought to the cardiac tissues. Fragmentation of muscle fibres arises due to severe inflammation bought by the induction of ethanol. More extensive necrosis is described as a focal degenerative myopathy which appears in the early stages of the disease. Similar observations were cited by Ferguson *et al.* (1986) in the cardiac tissues of Atlantic salmon. The same findings have been noted by Jittima *et al.* (2006) exhibited changes in the heart of hamsters (*Mesocricetus auratus)* when infected with *Leptospira interrogams* and serovar pyrogenes. Vacuolation refers to the swelling of the granules brought about by the presence of water which seems to get logged in the cells. Findings by Burkitt *et al.* (1996) supported this. Hyalinisation occurs when cells of cardiac tissue enters into a hypoxic state. The findings of Branka *et al.* (2005) support the same theory. Venkataramana *et al.* (2001) also observed the same changes in the cardiac muscle of the fresh water Gobiid fish, *Glossogobius giuris* (HAM) exposed to malathion. All the changes which occurred in *O. mossambicus* on exposure to ethanol for 7 days may be the result of impairment of smooth muscle fibers and an atrophy of cells in response to the physiological stress by ethanol induced toxicity. Cardiac muscle cells showing various degrees of degenerative and necrotic changes in their nuclei may be referred to as a sign of myocardial necrosis (Thomas, 1989). As with gills, cardiac muscle tissue also comes into close contact with the toxicant dissolved in water. This might result in fragmentation as well as vacuolation of sarcoplasm accompanied with necrosis. The findings of Fatma (2008) supported this. Jittima *et al.* (2006) cited similar changes in the heart of hamsters (*Mesocricetus auratus)* when infected with *Leptospira interrogams*, Serovar pyrogenes. Accumulation of leucocytes refers to the inflammation brought about by ethanol in the cardiac muscle of *O. mossambicus*. Observations made by Venkataramana *et al.* (2001) support the present finding.

This proves beyond doubt the statement of Mallat (1985) that toxicant induced histopathological changes are not specific for any class of chemicals and could be regarded as the result of patho physiological exposure of pollutants. This information about histopathology is helpful in the risk assessment of toxic compounds and also enhances knowledge of the pathophysiology of fish. It is thus concluded from the present study that exposure to different sub lethal concentrations of ethanol for 21 days followed by a periodical sampling at 7 days brings about severe damage to the vital organs of the fish which in turn could reflect perturbations of its physiology or biochemistry. Hence, environmental awareness is necessary about discharge of toxic pollutants into aquatic ecosystem. Further proper treatment of effluent is a necessary prerequisite for safe disposal into the aquatic system or environment.

Summary and Conclusion

With the global shortage in the production of fossil fuel and the ever increasing consumption of these, a major focus has developed worldwide on biofuel production. Ethanol seems to be a major fuel additive as well as a promising energy alternative. Ethanol has already been introduced on a large scale as a fuel additive in Brazil, USA, and some European countries, and it is expected to be one of the dominating biofuels in the transport sector in the next twenty years. Ethanol is being blended with diesel as well as with petrol. The production and usage of ethanol contributes to pollution of air, soil, water and global warming. Ethanol has also got the potential to transport the components of gasoline through ground water and surface water and to spread the floating product to a larger area due to the solvency of ethanol with gasoline components and water. Emergency Planning and Community Right Know Act (EPCKA) has considered ethanol as a hazardous chemical (USEPA, 2008).

The present study dealt with the haematological, biochemical and histopathological impacts of different sub lethal concentrations of ethanol on a euryhaline teleost *Oreochromis mossambicus* (Peters). Summary of the findings are listed below:

* When fishes were exposed to different lethal concentrations of ethanol, they exhibited erratic movements like initial increase followed by a decline in opercular movement, frequent surfacing and gulping, loss of equilibrium, grouping, increase in respiratory rhythm, excess secretion of mucus followed by a gradual shift to inactivity. These behavioural alterations themselves show the extent of the respiratory stress induced by ethanol.

* As per probit analysis, LC_{50} of ethanol on the test animal *O. mossambicus* was recorded as 13.107 g/l in which the 95% confidence limit ranged between 12.786 and 13.382 g/l. For conducting experimental studies sub lethal concentrations of ethanol which corresponds to $1/20^{th}$, $1/10^{th}$ and $1/5^{th}$ of the lethal concentration value was selected.

* Decrease in body weight was noted when *O. mossambicus* was exposed to ethanol for 7 and 21 days.

* It was confirmed through gas chromatography that 96% of ethanol is retained in the test medium in a 24 hour time period.

* By means of GC/MS method, it was established that about 0.34 µl of ethanol was present in 5 µl of a jaggery effluent which was almost equal to the sub lethal dosage value ($1/20^{th}$ of LC_{50}) indicating the potential toxic impacts of ethanol.

* Studies carried out using GC indicated an increase in blood ethanol concentration of the fish which mainly arose due to fishes entering into a state of hypoxia which explains ethanol production as an ubiquitous "anaerobic" end product, which gets accumulated whenever metabolic demand exceeds the mitochondrial oxidative potential. The very low amount of ethanol detected in the control group of *O. mossambicus* was mainly due to the activity of microorganisms in the gut of *O. mossambicus*.

* A significant decrease observed in the level of membrane bound enzymes in the gill tissues of *O. mossambicus* exposed to ethanol mainly refers to the damages in the membrane architecture which resulted in the non availability of substrates like ATP molecules leading to the inhibition of ATPase.

* Decrease in haemoglobin was found to be due to the reduction in the absorption of iron from the gut.

* Increase in the RBC Count mainly attributes to the hypoxic conditions. This was supported by the histopathological observation stating that there was epithelial damage of the gill lamellae.

* A decrease in the WBC count value was probably due to the result of increased secretion of corticosteroid hormones.

* The decrease in the PCV value obtained was attributed to the gill damage followed by impaired osmoregulation brought about by ethanol affecting the haemopoietic system and biochemical pathway of haem formation leading to microcytic anaemia.

* The decrease in the MCV value may be due to the release of immature red blood cells from haemopoietic tissues leading to the shrinkage of red blood cells resulting in microcytic anaemia.

* The significant increase in ESR rate indicates a possible pathologic condition.

* A subsequent increase in the levels of FPRC is due to an increase in the number of both immature and mature RBCs.

* The alterations brought about in the MCH, MCHC values may be attributed to a condition commonly associated with a decrease in number and increase in size and haemoglobin content of RBCs suggesting a hyperchromic anaemic state.

* An increase in RBC hemolysis observed in *in vitro* conditions refers to the membrane damage brought about by the direct effect of lipid peroxidation products which also confirms that ethanol brings about an increased disintegration of erythrocytes thus making RBC membrane more fragile.

* Increase in RBC hemolysis observed in *in vivo* conditions is due to the increased activities of serum specific enzymes. It also refers to membrane lipid peroxidation which in turn leads to changes in membrane fluidity, permeability and also results in the enhanced rates of protein degradation, eventually resulting in cell lysis. The decrease in hemolysis observed indicates that RBC membrane has become rigid. A fall in PUFA followed by an elevation of cholesterol increases the rigidity of the phospholipid bilayer.

* Decrease in serum copper and zinc levels indicated the disturbed immune system brought about by enhanced cholesterol, triglyceride synthesis and low density lipoprotein as well as decreased HDL levels. The significant decrease exhibited by serum selenium levels resulted from an increased protein damage brought about by an increased oxidative stress.

* Marked alterations exhibited in serum iron levels refer to the alterations in the iron content of the body. A significant increase in serum iron levels refers to increased iron absorption whereas significant decrease observed upon prolonged exposure points to the decreased iron bioavailability resulting in decreased iron absorption which may bring about alterations in the iron content of the body.

* An increase in serum creatine kinase values is due to the increased cardiac injury resulting in simultaneous elevation of creatine kinase levels.

* A significant increase in serum ALT and AST levels explains that during hepatobiliary disorder, amino acids are released from damaged tissues. In order to metabolize these amino acids, the process of transmutation gets enhanced leading to increased activity of the related enzymes such as AST and ALT.

* A significant increase in serum ALP activity has been related to tissue damage leading to the release of ALP into the blood.

* Decrease in protein points to the degradation of protein into free amino acids which were obtained under stress condition suggesting the inhibitory effect of ethanol at transcription /translation levels.

* An increase in the serum cortisol value is attributed to increased metabolic activity brought about by the intake of the toxicant ethanol.

* A significant decrease in serum folic acid is due to folic acid malabsorption.

* An increase in the serum vitamin B12 is due to the release of vitamin B12 from the ethanol-damaged liver into the circulation.

* An increase in serum ferritin level mainly points to the severity of liver damage which resulted in the elevation of serum ferritin level.

* Increased blood glucose level indicates increased breakdown of glycogen to glucose resulting in its mobilization to other tissues in order to meet the energy crisis.

* An increase in serum LDH activity indicates the liver destruction brought about by ethanol resulting in the release of the enzyme into the blood which resulted in switching on to anaerobic respiration mainly to meet the energy demands when aerobic oxidation was lowered.

* The decrease in the total carbohydrate content in the tissues refers to the rapid utilization of carbohydrates by the tissue, possibly to overcome the stress which suggests the tendency of the metabolism of carbohydrates to shift more towards anaerobic dependence than aerobic oxidation through Kreb's cycle.

* Decrease in Cyt.c oxidase activity in the tissues results in the reduced availability of oxygen, which in turn has reduced the capacity of the electron transport system to produce ATP molecules.

* Decrease in serum albumin level reflects disturbances in liver function integrity.

* Decrease in serum urea level is due to the inhibition of enzymes in the uricolytic pathway.

* The significant increase in serum uric acid and creatinine values refers to glomerular insufficiency, increased muscular tissue catabolism as well as decreased urinary clearance by the kidney.

* The increase in the serum ammonia level points to increased protein catabolism.

* The increase in tissue total protein level in liver and kidney tissues is due to increased biosynthesis of proteins brought as a result of stress. The decrease in the total protein content in gills, muscle and heart refers to the protein degradation which simultaneously results in an elevation in the free amino acid levels.

* An increase in acid phosphatase enzyme was observed both in the serum and tissues of *O. mossambicus*. The increase in the serum activity of ACP is an indicative of leakage of ACP from the tissues to the serum. The increase in the ACP activity in the tissues as observed in the present study is due to the interaction between the ethanol and the cells of the tissues which increases the ACP activity and results in the leakage of ACP from the tissues to the serum.

* HMG CoA reductase activity determined by a ratio method (HMG CoA / Mevalonate) showed a significant decrease in all the tissues indicating increased cholesterogenesis.

* The increase in lipid content in serum and tissue indicates increased lipogenesis.

* A significant increase obtained in the serum and tissue phospholipid levels, points to the non-utilization of this lipid by the tissues.

* *O. mossambicus* entering into a state of hypoxia brings about a significant decline in the free fatty acid levels in the serum and muscle tissues.

* The increase in serum lipase activity refers to injury of the pancreatic acinar cells which results in the leakage of this enzyme into the blood leading to hyperlipasemia.

* A decrease in serum triglyceride value refers to the increase in the activity of lipase enzyme which could probably result in the increased uptake of circulating triglycerides leading to lowering of serum triglyceride level. The elevation in the levels of tissue triglycerides may be attributed to enhanced triglyceride synthesis or to reduced triglyceride catabolism.

* An increase in the HDL- cholesterol level in the serum of *O. mossambicus* refers to the increased lipoprotein lipase activity which results in the enhanced transfer of surface components (such as cholesterol and phospholipids) from triglyceride rich lipoproteins to HDL which mainly arises during lipolysis.

* A significant increase in the LDL+ VLDL values was observed in the serum, of *O. mossambicus.*

* The decrease in the serum cholesterol value may be due to the increased activity of plasma lecithin cholesterol acyl transferase. The elevation of the tissue cholesterol level may be attributed to enhanced cholesterol synthesis or due to reduced cholesterol catabolism.

* The increase in tissue MDA, CD and hydroperoxides followed by a decrease in SOD and CAT activity refers to the increased generation of reactive oxygen species (ROS) arising due to the excessive oxidative damage resulting in the production of free radicals arising due to oxidative stress.

* The decrease in GPX and GST observed during ethanol toxicity studies is due to their increased utilization to scavenge the significantly elevated levels of ROS

that has been formed. The non availability of glutathione decreases the activity of glutathione peroxidase and glutathione - S - transferase.

* The decrease in G-6-PD suggests decreased supply of NADPH. The decrease in all the enzymes indicates severe impairment of the tissues resulting in the inhibition of enzymes during ethanol treatment.

* A decrease in reduced glutathione level is related to the increased lipid peroxidation. Decreased GR activity may be a predominant cause for GSH depletion.

* The decrease in peroxidase activity is expected to reduce the level of ROS by metabolizing H_2O_2 which mainly points to inhibition of enzyme synthesis.

* The decrease in the non enzymatic antioxidant parameters such as GSH, vitamin C and vitamin E arise due to the increased turnover, for preventing oxidative damage suggesting an increased defense against oxidant damage.

* The decrease in vitamin C is due to the decrease in catecholamine release from the adrenal medulla which counteracts the fall in vitamin C owing to oxidative stress.

* The decrease in vitamin E refers to the increased free radical generation.

* The decrease in total SH groups are mainly related to the decrease brought about in membrane ATPase activity.

* The decrease in vitamin A level mainly arises due to increased mobilization of vitamin A from the liver brought about by increased catabolism of vitamin A in the liver or in other organs.

* Decrease in lysosomal fraction of β-glucuronidase and acid phosphatase activities both in *in vitro* studies and *in vivo* studies are usually associated with a reduction in the stability of lysosomal membranes making them more fragile

resulting in an increased release of acid hydrolases posing a potential risk to the health of the cell leading to death of the cell or of the individual organism.

* Light microscopic examination of gill tissues of *O. mossambicus* exhibited the pathological condition like hyperplasia of epithelial cells, which serves as physical and physiological defense mechanism which in turn increases the diffusion distance thus affecting the exchange of gases. Oedema observed in the present study, is a defense mechanism that reduces the branchial superficial area of the fish which comes in contact with the external milieu. These mechanisms also increase the diffusion barrier to the pollutant. Dilation of the blood vessels is due to increased permeability helping in the free passage of ethanol into the blood stream. Telangiectasis observed explains the state of asphyxia of the fish when subjected to ethanol toxicity indicating acute respiratory distress. Gill aneurysm observed indicates impaired respiratory function. This is related to the rupture of the pillar cells which results in an increased blood flow inside the lamellae, causing dilation of the blood vessel or even aneurysm of gill. Haemorrhages, hyperplasia and clubbing reduce the respiratory area thereby reducing the respiratory and osmoregulatory potential of the fish. It also indicates a decrease in energy metabolism due to the degeneration of respiratory epithelium and the damage brought to the gill tissue finally result in tissue hypoxia. These pathological changes observed in respiratory organs might have resulted in a shift from aerobic to anaerobic pathway of metabolism in the tissues of fish under pollutant stress. The clubbed appearance of lamellae refers to lamellar hyperplasia from where cells are derived from primary lamellae and migrate towards the distal end. This finally results in accumulation of cells at the leading edge of secondary lamella, which colloquially is termed as 'clubbing' of lamellae. Necrosis of the gills, haemorrhages followed by complete epithelium desquamation have been considered as a common reaction towards strong water pollution arising due to the presence of ethanol. Haemorrhages can be interpreted as a reflection of the direct action of ethanol on the tissue. These changes were referred to as by-products of catabolism.

* Light microscopic examination of liver tissues of *O. mossambicus* exhibited the pathological condition like ceroid pigmentation mainly brought about by the free radical induced damage to the hepatocytes. Focal area of necrosis observed has resulted from the excessive work brought about by the fish to get rid of the toxicant from its body during the process of detoxification. It might have also developed by the inability of the fish to regenerate new liver cells. Fatty changes observed are the characteristic feature of liver damage. The changes in fat content may be due to the increased mobilization and transport of fat to the liver. Fatty changes are the characteristic feature of liver damage. Marked histopathological changes such as hepatic cord disruption, pyknotic nuclei, extensive proliferation of the biliary epithelium and ceroid pigmentation were noted. Hepatocyte necrosis was brought about by the wide spread inflammation as well as destruction of the liver brought about by the increased concentration of ethanol. Extensive proliferation of the biliary epithelium in the liver revealed that the metabolic storage products were affected. The occurrence of necrosis is said to be one of the most visible damages observed in liver tissues when subjected to ethanol toxicity which arises as a consequence of enzymatic inhibition, damages in the cellular membrane integrity, and disturbances in the synthesis of proteins and carbohydrate metabolism. Histopathological changes such as hepatic cord disruption, pancreatic tissue necrosis, hepatocyte necrosis and proliferation of bile duct tubules were found. This has been brought about by tissue hypoxia. Necrosis found in parenchymatous tissue is brought about by the liquefaction of hepatocytes.

* Light microscopic examination of kidney tissues of *O. mossambicus* exhibited the pathological condition such as vacuolation of epithelial cells, thickening of Bowman's capsule, shrinkage of glomeruli and adhesion which refers to that of glomerulonephritis. Vacuolation of the epithelial cells in the kidney has been demonstrated to be a morphological change consistent with potassium depletion that is a feature of the diuretic stage of acute tubular necrosis. Loss of tubules refers to the signs of acute tubular necrosis. Thickening of Bowman's capsule as well as intercapillary thickening is related to the disturbances of the ionic state of the cells caused by the toxicant ethanol. Fibrosed area followed by tubular

necrosis occurs as a result of a degenerative process taking place in the tissue. Fibrosed area also refers to the proliferation of fibroblasts occurring in kidney tissues.

* Light microscopic examination of heart tissues of *O. mossambicus* exhibited the pathological condition like loss of striation, vacuolation of sarcoplasm, phagocytic accumulation, loss of muscle fibres, more extensive necrosis, hyalinization, fragmentation of muscle fibres, leucocyte accumulation at the periphery, destruction of muscle fibres, fragmentation as well as necrosis. Loss of striations, leucocyte accumulation followed by necrosis refers to the indications of damage bought to the cardiac tissues. Fragmentation of muscle fibres in heart arise due to severe inflammation bought by the induction of ethanol. More extensive necrosis is described as a focal degenerative myopathy which appears in the early stages of the disease. Vacuolation refers to the swelling of the granules brought about by the presence of water which seems to get logged in the cells. Hyalinization occurs when cells of cardiac tissue enters into a hypoxic state. Cardiac muscle cells showing various degrees of degenerative and necrotic changes in their nuclei are referred to as a sign of myocardial necrosis. As with gills, cardiac muscle tissue also come into close contact with the toxicant dissolved in water. This might result in fragmentation as well as vacuolation of sarcoplasm accompanied with necrosis. Accumulation of leucocytes refers to the inflammation brought about by ethanol in the cardiac muscle of *O. mossambicus*. The pathological changes observed revealed that they were dependent upon the time and dosage.

* The present findings warrant future studies to explore ATPases as possible biomarkers of pollutant exposure in ecotoxicology. This study indicated that *O. mossambicus* when exposed for 7 and 21 days to ethanol was under tremendous stress and parameters employed in this study can be adapted for future investigations as biomarkers of damage caused by ethanol to aquatic organisms. The present study revealed that *O. mossambicus* is sensitive to sub lethal concentrations of ethanol.

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List of publications

Research Papers

1. **Smitha.V.Bhanu,** Babu Philip, Aniladevi Kunjamma KP and Jisha Jose (2009). Effect of ethanol on haematological parameters of *Oreochromis mossambicus* (Peters). *Pollution Research*, Accepted for publication in Dec (2009), Issue No: 4.

2. Aniladevi Kunjamma KP, Babu Philip, **Smitha.V.Bhanu** and Jisha Jose (2008). Histopathological effects on *Oreochromis mossambicus* (Tilapia) exposed to Chlorpyrifos. Journal of Environmental Research and Development, 2(4): 553-559.

Seminar Presentations

National conference

1. **Smitha.V.Bhanu,** Babu Philip, Aniladevi Kunjamma KP and Jisha Jose (2009). Effect of ethanol on haematological parameters of *Oreochromis mossambicus* (Peters). 18th Swadeshi Science congress, Book of Abstracts, LS-11.

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3. Aniladevi Kunjamma KP, Babu Philip, **Smitha.V.Bhanu** and Jisha Jose (2008). Effect of Chlorpyrifos on branchial – ATPase enzymes of *Oreochromis mossambicus* (Peters), International Conference on bio-diversity, conservation and management, theme: Ecosystems and sustainable development, BIOCAM 2008, Book of Abstracts, EDB-08.