# BIOCHEMICAL EFFECTS OF PETROLEUM HYDROCARBONS ON THE TROPICAL TELEOST *OREOCHROMIS MOSSAMBICUS* (PETERS)

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BY


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*To God Almighty*

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# Certificate

This is to certify that the thesis entitled **Biochemical Effects of Petroleum Hydrocarbons on the Tropical Teleost *Oreochromis mossambicus* (Peters)** is an authentic record of the research work carried out by **Ms. Jehosheba. P. Mathews** under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfilment of the requirements of the degree of **Doctor of Philosophy** in **Biochemistry** of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any university.

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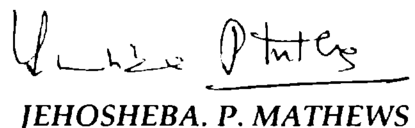
# DECLARATION

I hereby declare that the thesis entitled **Biochemical Effects of Petroleum Hydrocarbons on the Tropical Teleost *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.



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*Kochi-16* *Jehosheba P Mathews*
*November 2003*

## *List of Notations and Abbreviations*

| | |
|---|---|
| ACP | acid phosphatase |
| ADP | adenosine diphosphate |
| ALT | alanine transaminase |
| ANOVA | analysis of variance |
| AST | aspartate transaminase |
| ATPase | adenosine triphosphatase |
| CAT | catalase |
| CD | conjugated dienes |
| CDNB | 1-chloro 2, 4-dinitrobenzoic acid |
| °C | degree centigrade |
| ē | electron |
| EDTA | ethylene diamine tetra acetic acid |
| g | gram |
| GPX | glutathione peroxidase |
| GR | glutatione reductase |
| GSH | Glutathione (reduced) |
| GSSG | oxidised glutathione |
| GST | glutathione-S-transferase |
| h | hour |
| ˙OH | hydroxyl radical |
| $H_2O_2$ | hydrogen peroxide |
| $H_2SO_4$ | sulphuric acid |
| Hb | haemoglobin |
| IU | international unit |
| $LC_{50}$ | lethal concentration causing 50% mortality |

| | |
|---|---|
| LSD | least significant difference |
| M | Mole, Molar |
| MDA | malondialdehyde |
| µg | microgram |
| µl | microlitre |
| mg | milligram |
| ml | millilitre |
| mM | millimoles |
| N | normal |
| NAD | nicotinamide adenine dinucleotide |
| NADH | reduced nicotinamide adenine dinucleotide |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NaOH | Sodium hydroxide |
| nm | nanometer |
| $O_2$ | oxygen |
| $^{\cdot}O_2^{-}$ | oxygen free radical |
| PCV | packed cell volume / haematocrit |
| PHC | petroleum hydrocarbons |
| PUFA | poly unsaturated fatty acid |
| RBC | red blood corpuscles |
| SOD | superoxide dismutase |
| TBA | thiobarbituric acid |
| TCA | trichloro acetic acid |
| WAF | Water- accommodated fractions |
| WBC | white blood corpuscles |

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# *Chapter* 1

# Introduction

## 1A GENERAL INTRODUCTION

Spiralling increases in world population have stimulated concern among food scientists about the rapidly dwindling supply of animal protein. The oceans contain virtually untapped food sources, yet these ecosystems are threatened by man and his technology. One of these threats is the result of increased oil production, transportation and refining as the petroleum industry attempts to meet increased demands caused by energy crisis. The use of supertankers now makes accidents at sea a much greater hazard than formerly, as the oil contained in a supertanker can adversely affect tremendous areas of the sea (Anon, 1991). About 50% of the world's crude oil production is carried by tankers. The amount of petroleum hydrocarbons entering the oceans is increasing as a function of this increased transportation. In addition to tanker spills, the amount of petroleum hydrocarbons entering the oceans from offshore and refinery operations is also increasing. In the order of 5 million tons of crude oil from a variety of sources is estimated to enter the marine environment each year and as a result, the occurrence of petroleum-derived hydrocarbons in contaminated marine coastal waters is now up to 80ppb with occasional records of 500ppb in the Arabian Gulf (Hinrichsen, 1990). After any spillage of crude oil, a number of simultaneous processes occur: spreading, dispersion, volatilization, evaporation, photooxidation, emulsification, sedimentation and biodegradation which together determine the fate of the constituent hydrocarbons.

Crude oil makes up 53% of the petroleum hydrocarbons entering the oceans. The remaining 47% consists of diesel oil, gasoline and other residual oils. Crude oil, an extremely complex mixture of hydrocarbons is divided into three major classes: alkanes, cycloalkanes and aromatics. The aromatic hydrocarbons (benzene, toluene, xylene, phenols, quinolines, naphthalenes, phenanthrenes, alkyl phenols, anilines, indoles, benzothiophenes etc) are the most toxic of the three and the most water soluble fractions of crude oil, (Blackman *et al.*, 1980; Winters and Parker, 1977) more toxic than alkanes. Spills into deeper waters or very large spills may result in significant dissolution of aromatic hydrocarbons as oil floats to the surface, or beneath a thick oil slick. Thus, in the immediate aftermath of such spills, dissolved aromatic hydrocarbons may form an acutely toxic mix (Singer *et al.*, 2000). The toxicity of petroleum is mostly related to its water-accommodated fractions (WAF) that contain, among other organic and inorganic compounds, the short-chain polycyclic aromatic hydrocarbons (PAH), which occur in low concentrations, but are ubiquitous environmental pollutants and can be environmentally dangerous. The less soluble fraction of crude oil is composed of long chain hydrocarbons persisting for long time in the environment and posing a physical threat to fish creating a viscous barrier at the water-air interface. On the other hand, water-accommodated fractions (WAF) which contain mono- and polycyclic aromatic hydrocarbons may cause biochemical disturbances in the organisms (Bobra *et al.*, 1989; Markarian *et al.*, 1995). It is well documented that even 1 mg/l of oil dispersed in sea water or 1µg/l of water-soluble oil components can harm aquatic organisms (Shugart, 1988).

The present work is a base-line attempt to investigate and assess the toxicity of water-accommodated fractions (WAF) of Bombay High crude oil. The experimental animal selected for the present study is a euryhaline teleost, *Oreochromis mossambicus* (Peters), adapted to fresh water. The fish has been selected on account of its economic valve, abundant availability, experimental feasibility, ease of rearing and maintenance and also because it is one of the commonly cultured species in the South-East Asian countries.

All investigations presented in this thesis are aligned into eight chapters with the following objectives.

1 To determine the effects of water-accommodated fractions (WAF) of crude oil on carbohydrate metabolism.

2. To study the effects of WAF of crude oil on nitrogen metabolism.

3. To assess and evaluate the effects of WAF of crude oil on lipid peroxidation.

4. To examine the effects of WAF of crude oil on lysosomal membrane stability and some haematological parameters.

5. To document the effects of WAF of crude oil on branchial $Na^+$-$K^+$ATPase and bioaccumulation of petroleum hydrocarbons in different tissues.

6. To examine the histopathological changes in gill tissue.

## 1B. REVIEW OF LITERATURE

Crude oil or petroleum a transformation product of fossil (often marine organisms) is a liquid mixture, occurring underground, of many thousands of organic compounds amongst which hydrocarbons predominate. This mixture contains compounds ranging from low-molecular-weight hydrocarbons to very complex polynuclear aromatic hydrocarbons (PAH) containing numerous isomers. In addition to hydrocarbons, sulphur, oxygen and nitrogen derivatives of hydrocarbons are also present in oils in varying proportions, as are complexes of organically bound nickel, vanadium and cobalt. Nickel and vanadium are often present in µg/g quantities (NRC, 1985) and their ratios can be used to characterize a crude oil.

### 1B.1 Solubility of crude oil in water

The specific gravity of whole crude oils usually is less than 1.0, i.e. they float on water. Aqueous solubility depends on the structural type, with normal alkanes being least soluble and aromatic hydrocarbons being most soluble in water (Lysyj *et al.*, 1980). Solubilities in water are also inversely proportional to molecular weights (McAuliffe, 1980). Therefore, the fraction of a natural water body, dissolved in it has a composition different from the original oil, with an enrichment of the water- soluble low-molecular-weight aliphatic and aromatic components (Shiu *et al.*, 1990). Studies have suggested that the toxicity of oil is primarily due to the water-soluble fraction (Rice *et al.*, 1977). The light aromatics (benzene to naphthalene) are considered to be one of the most immediately toxic components of petroleum other than the carcinogenic polycyclic aromatic hydrocarbons (PAH).

Behavior and distribution of PAH in the environment and therefore, the effects on biological systems vary due to differences in molecular weight. The low molecular weight PAH i.e., 2-3 ring aromatics (naphthalenes, fluorenes, phenanthrenes and anthracenes) have significant acute toxicity to aquatic organisms, whereas the high molecular weight PAH i.e., 4-7 ring aromatics (chrysene-coronene) do not (Neff, 1979). Additionally the current theory of a narcosis mechanism of toxicity (non-specific toxicity) suggests that all soluble components of oil bioaccummulated in aquatic organisms may contribute to toxicity (Peterson, 1994; Barron *et al.*, 1999).

When crude oil is mixed with water, a much more physically and chemically complicated mixture is formed. The bulk of the oil is mostly non polar and forms into spheres of various sizes. Compounds then partition between the oil droplets and the water (Shaw, 1977). It has been shown that crude oil droplets in water are coated by a layer of water molecules (Malcolm and Commaert, 1981) which prevents the droplets from coalescing on contact with each other. Apart from tending to keep oil in an emulsion, these water jackets on the oil droplets could be important in increasing contact between oil and biological components such as mucus.

### 1B.2 Physiological and behavioural responses

Blumer (1969) was the first to suggest that exposures to petroleum hydrocarbons could interfere with chemoreception and this could result in altered behavioural patterns. A reduction in feeding rate of *Mytilus edulis* is known to occur at low and environmentally realistic concentration of petroleum hydrocarbons (30-40 µg $l^{-1}$) (Widdows *et al.*, 1984). Probably due

to olfactory disturbances, it was observed that feeding rate in presence of petroleum hydrocarbons was reduced. Widdows *et al.* (1985) have also demonstrated a reduction in the efficiency of food absorption by *M.edulis* under petroleum hydrocarbons stress. Behavioural and physiological responses observed during short-term exposure to hydrocarbons may be restored to control levels following transfer to uncontaminated water although recovery does not always occur immediately upon transfer (Capuzzo *et al.*, 1984). The inhibition of feeding results from the narcotic effect of hydrocarbons, particularly aromatic hydrocarbons, which may have a direct action on cilia and muscles or the nervous system which control such activity (Hendry *et al.*, 1985). *M.edulis* exposed to 130 µg $l^{-1}$ diesel oil for 8 months showed a marked reduction in the feeding rate and a negative scope for growth indicating the need to utilize body reserves in order to satisfy the animal's energy requirements (Widdows *et al.*, 1985). McCain *et al.* (1978) suggested that, flat fish exposed to oiled sediment reduced food intake. Gilfillan *et al.* (1976) reported a similar type of growth reduction and increased respiration in *Mya arenaria* following Bunker crude oil exposure.

Respiration was largely affected in presence of petroleum hydrocarbons. Rate of oxygen consumption has been used as a valuable tool by many workers to assess stress, since it is an index of energy expenditure to meet the demands of environmental alterations (Prabhudeva and Menon, 1986). Exposure to petroleum hydrocarbons generally tends to increase respiratory rate (Bayne *et al.*, 1985). The investigations by Correa and Garcia (1990) on the effects of benzene on the physiological responses of juvenile white mullet, *Mugil curema*, have proved that benzene modifies the gaseous

exchange and therefore, has an influence on the respiratory rate. Low levels of petroleum hydrocarbons are known to enhance respiratory activities in *M.edulis* and *Mya truncata* (Hutcheson, 1982). The deleterious effect of crude oil is due to a film formed over the gill filaments preventing the exchange of gases and resulting in anoxia and suffocation. The most profound physiological disturbance in fishes exposed to water-soluble fractions of crude oil was a dramatic decline in blood oxygen content (73% decrease), after 48-h exposure, which is likely to be the cause of the increased plasma noradrenaline (Alkindi *et al.*, 1996).

Chipman and Galtsoff (1990) studied the effects of crude oil on oysters and found that water-accommodated fractions reduced the number of hours the test oysters remained open in comparison with the control and that the rate of water transport through the gills decreased from 207-310 l /day to 2.9-1.0 l /day between the eighth and $14^{th}$ day.

Chronic exposure of cod to 150-300ppb of Venezuelan crude and as little as 50ppb of Hibernia crude causes enlargement of the heart (up to 50%) (Khan *et al.*, 1987). The heart enlargements is probably due to the damage to gill lamellae, which results in increased gill resistance, thus increasing the load on the heart. Similarly gall bladder size is also affected by oil exposure in cunner (Kiceniuk *et al.*, 1980), winter flounder (Fletcher *et al.*, 1982), and sculpirts (Kiceniuk *et al.*, 1982).

Percy and Mullins (1977) found that exposure to even low concentrations of crude oil significantly impaired the locomotary activity of the amphipod, *Onismus affinis* which could have an impact on the survival of

affected populations in nature. Altered patterns in swimming speed, pause intervals between swimming and allocation in time to active food search were seen in the copepod, *Centropages hamatus* exposed to sublethal concentrations of crude oil (Cowles, 1983).

Hedtke and Puglisi (1980) found that egg production was impaired in American flagfish on exposure to 338 μl /l of the water-soluble fraction of the crude oil. Kiceniuk (1982) observed that testes somatic indices in cod exposed to 50-100ppb of Hibernia crude oil in sea water during the early stages of gonad maturation were reduced sevenfold relative to control fish. *M.balthica* exposed to 30 μg petroleum hydrocarbon $l^{-1}$ also showed gamete resorption and abnormal gamete development at 300 μg $l^{-1}$ (Stekoll *et al.*, 1980).

### 1B.3 Biochemical studies on petroleum hydrocarbons

Gagnon and Holdway (2000) studied the effects of water-accommodated fractions of Bass Strait crude oil on 6 days exposure on immature Atlantic Salmon (*Salmo salar*). It was noted that serum sorbitol dehydrogenase activity was decreased below the normal level, indicating liver damages. An increase in hepatic 7-ethoxyresorufin O-deethylase (EROD) activity was also observed.

The effect of crude oil exposure on blood phosphate levels and ion regulation in an air-breathing teleost fish *Hoplosternum littorale* was studied by Brauner *et al.* (1999). The results indicate that a single oral dose of 3.0ml/kg of Urucu crude oil elevated net whole body $Na^+$ efflux and resulted in a 7% reduction in plasma [$Na^+$] 72 h following ingestion, whereas reduction

in plasma [$K^+$] was noticed after 24 h ingestion. They also observed that 24% reduction in ATP· Haemoglobin ratio from (0.206 to 0.157) and a 31% reduction in GTP  Haemoglobin ratio (0.455 to 0.315) 24 h following ingestion indicating that these fish may be hypoxemic, thereby it affects gas exchange and ion regulation.

Toxicity of Venezuelan crude oil for 4-5 months duration was studied in winter flounder by Fletcher *et al.* (1982). The results indicated that the enlarged livers of the oil exposed flounder had reduced concentrations of DNA, protein, $Na^+$ and $Zn^{2+}$ and increased concentrations of lipid and phospholipids. The reduced DNA and $Na^+$ concentrations suggested liver hypertrophy rather than hyperplasia. The increased phospholipids concentrations also suggested growth of membrane structures such as endoplasmic reticulum.

Influence of water-soluble fractions of southern Louisiana crude oil on the biochemical composition and enzymes of Atlantic Croaker, *Micropogon undulates L.* was studied by Jo Ann Eurell and Haensly (1981). The exposure was for 1, 3, 7, 14 and 21 days. Increases in glucose-6-phosphate dehydrogenase activity and levels of lipid and cholesterol with simultaneous decrease in glycogen were noted. Similar depletion in energy reserves was also observed by Duncan *et al.* (2002) in *Colossoma macropomum* when exposed to petroleum hydrocarbons. These observations support a hypothesis that exposure to petroleum hydrocarbons increases mixed function oxidase activity.

Prasad *et al.* (1987) observed that exposure of cat fish, *Heteropneustes fossilis* to different concentrations of crude oil for a varying period resulted in hyperglycemia. Similar results have been reported by Alkindi *et al.* (1996) in flounders, *Pleuronectes flesus* as well as by Omoregie (2002) in Nile tilapia, *Oreochromis niloticus L.*

Effect of petroleum hydrocarbons on the biochemical changes of *Australian Bass* and *Macquaria novemaculeate* were studied by Nugegoda (2001) and observed that the lactate dehydrogenase(LDH) activity was increased indicating a shift towards anaerobic metabolism. Similarly Omoregie (1997) found stimulation of LDH activity in tissues of Nile tilapia treated with petroleum hydrocarbon effluent. Chronic exposure of Empire Mix crude oil resulted in decrease of oxidative enzymes-succinate dehydrogenase and malate dehydrogenase in homogenates of American oysters (*Crassostrea Virginica*) (Janice *et al.*, 1979).

More recent evidence indicates that cytochrome-c-oxidase was significantly inhibited by petroleum hydrocarbons (Cohen *et al.*, 2001) and decrease in cytochrome-c-oxidase activity suggests mitochondrial electron transfer system to be affected under petroleum hydrocarbons impact, leading to reduced synthesis of ATP molecules. Brown *et al.* (1996) reported similar type of inhibition of cytochrome-c-oxidase in *Clupea Pallasi* by Exxon Valdez oil impact.

Dange *et al.* (1985) studied the effect of toluene, a major water-soluble and toxic component of various crude oils for 10 weeks in different tissues of *O.mossambicus*. Transaminases are among the crucial enzymes

in amino acid metabolism, which in aquatic organisms are known to be affected by exposure to oil hydrocarbons. There was considerable increase in AST and ALT activities. Narvia (1997) also showed similar increase in transaminase in *Mytilus edulis L.* In response to exposure to high concentrations (>1 mg $l^{-1}$) of petroleum hydrocarbons there was an elevated rate of ammonia excretion in marine snail, *Thais lima* and in bivalve, *Venus verrucosa* reflecting enhanced utilization of protein reserves for maintenance and survival (Stickle *et al.*, 1984 and Axiak and George (1987)).

The alkaline phosphatase plays an important role in phosphate hydrolysis, transport of sugars etc. There are some reports on the effect of petroleum hydrocarbons on this enzyme. Janice *et al.* (1979) investigated the effects of Empire Mix crude oil on alkaline phosphatase and found that ALP activity was increased in American oysters as well as in Brown shrimp.

Dev *et al.* (1983) studied that the influence of crude petroleum on liver lipids and fatty acids in cod, *Gadus morhua* and winter flounder, *Pseudopleuronectes americanus*. The parameters studied included free fatty acids, total phospho lipids, tri glycerides and essential fatty acids. There was a significant increase in the levels of total phospho lipids and free fatty acids whereas significant decrease in triglycerides was observed in experimental animals. These observations strongly indicate rapid mobilization and utilization of stored pool of lipids. Similarly Krishnakumar *et al.* (1997) found an increase in neutral lipid in the digestive tissue of *Mytilus edulis* exposed to microencapsulated mixture of petroleum hydrocarbons (composed of phenanthrene, fluoranthene and benzo (a) pyrene).

Wang and Stickle (1988) found that there was a reduction in the total protein, lipid and RNA content in juvenile blue crab, *Callinectes sapidus* when exposed to water-soluble fraction of South Louisiane crude oil (0-2500ppb) for 21 days. The results also indicate that the ratios of RNA: DNA and protein: DNA decreased in crabs exposed to crude oil.

Petroleum hydrocarbons or their metabolites can directly or indirectly affect the genetic material as indicated by a number of observations at different levels of genetic and cellular organization. Neoplastic diseases found in bivalves have a definite correlation with exposure to petroleum hydrocarbons (Mix *et al.*, 1979; Brown *et al.*, 1979). Epithelial tumors were induced in the gastropod, *Ampullarius australis* by exposure of petroleum hydrocarbons (Krieg, 1972). Embryo abnormalities occur in the periwinkle, *L. saxatilis* in association with the presence of petroleum hydrocarbons (Dixon *et al.*, 1985). Chromosomal aberrations, in the form of increased aneuploidy, were found in *M.edulis* with high burden of petroleum hydrocarbons (Dixon, 1982). Carcinomas and papillomas were found on the lips of croakers, a bottom feeding fish caught in Pacific Ocean in an area polluted with carcinogenically potent wastes released from a nearby oil refinery. Young (1974) observed papillomas on the body and lips of several types of fish, including the white seabass (*Cynoscion nobilis*), the Dover soles (*Microstromus pacificus*), the white croakers (*Genyonemus lineatus*) and Pacific sandcrabs *(Citharichthy sordidus)* from two oil-polluted areas. Tumerous lesions were produced experimentally on the killifish (*Fundulus porvipinnis*) in 12 days, and the fish died shortly thereafter. These tumours, some of which were cancerous, were never observed on fish taken from

unpolluted waters. Ahokas *et al.* (1979) reported a high incidence of tumors in bottom fish from the Buffalo River contaminated with petroleum hydrocarbons.

The effects of petroleum hydrocarbons on gill osmoregulatory function were studied by monitoring the changes in the activity of the gill $Na^+$-$K^+$ATPase. The effects of WAF of crude oil on ATPase activity was studied by Wong and Engelhardt (1984), in rainbow trout *(Salmo gairdneri)*. They exposed the animals to sublethal concentrations of WAF The analysis of the enzyme activity revealed that $Na^+$-$K^+$ATPase enzyme activity was significantly inhibited. Studies of McCloskey and Oris (1993) also revealed that exposure of bluegill sunfish to anthracene decreases the specific activity of branchial $Na^+$-$K^+$ATPase. The inhibitory effect on $Na^+$-$K^+$ATPase is generally ascribed to structural and functional damage to the gills as a result of accumulation of petroleum hydrocarbons (Pelgrom *et al.*, 1995).

Eales *et al.* (1993) studied that the effect of crude oil on hormonal balance. Exposure of flounder to water-soluble fractions of crude oil caused a more marked decline in plasma $T_4$ concentrations than that occurring in control fish. The narcotic action of volatile hydrocarbons (perhaps together with the low blood oxygen) may have depressed hypothalamic release of thyroid releasing hormone (TRH) or pituitary release of thyroid stimulating hormone (TSH), and thus further depressed plasma $T_4$ concentrations of these fish (Leatherland *et al.*, 1990). The release of catecholamines, especially noradrenaline apparently in response to the very low blood oxygen content of flounders exposed to water-soluble fractions of crude oil was observed by Kita and Itazawa (1989). Laboratory stimulation studies were

conducted by Alkindi *et al.* (1996) to assess the impacts of crude oil, on level of cortisol. There was a significant increase in plasma cortisol concentrations in experimental flounders exposed to water-soluble fractions of crude oil.

Exposure of marine mollusks to petroleum hydrocarbons has been demonstrated to result in increases in the activities of certain lysosomal enzymes, notably, β-glucuronidase and acid phosphatase (Moore *et al.*, 1982). Other lysosomal contents such as lipofuscin have also been observed to accumulate following exposure to petroleum hydrocarbons in several species of mollusk (Moore *et al.,* 1985). Lipofuscin or aging pigment is a product of free-radical peroxidative reactions derived from the autophagy of lipoprotein membranes (Brunk and Collins, 1981).

Haematological investigations on crude oil toxicity in fish blood were done by Klyszejko (1982) in *Anguilla anguilla L.* The sublethal exposure resulted in decrease in the RBC count, WBC count and haemoglobin. Petroleum hydrocarbons induced blood anaemia has been observed in experiments with fresh water cat fish, *Heteropneustes fossilis* (Prasad *et al.*, 1987).

### 1B.4 Histological studies on Petroleum hydrocarbons

Many histological studies were also reported on petroleum hydrocarbons toxicity in fishes. Woodward *et al.* (1983) studied the histopathological effects of crude oil on fishes at the light microscopic level and observed that the changes associated commonly with the gills were lesion, oedema and mucous cell hyperplasia. Fletcher *et al.* (1982) noted a reduction in the size of the testes in male *P americanus* exposed to oil

sediments, the probable reason being the increase in metabolic demands of the fish by the oil thereby requiring them to utilize the testes as energy sources. Histological analysis of mussels (*M. edulis*) from petroleum hydrocarbon-polluted sites indicates gamete degeneration of the germinal tissues (Lowe, 1988).

Sunila (1988) correlated the occurrence of inflammatory reactions, ulcers and haemorrhages of the digestive tract and kidney lesions in *M.edulis* with the toxicity of petroleum hydrocarbons.

Haensly *et al.* (1982) studied the changes induced in different tissues of *Pleuronectes platessa L. Pleuronectes platessa L.* by exposure to Amoco Cadiz crude oil. The histopathological alterations were characterized by fin and tail necrosis, hyperplasia and hypertrophy of gill lamellar mucous cells, gastric gland degeneration, decreased hepatocellular vcuolation (lipid), increased concentrations of hepatic macrophage centres and lateral trunk muscle fiber degeneration. Dilation of Bowman's space, glomerular hypertrophy and abdominal muscle fiber degeneration were also pronounced during long term exposure.

Effects of chronic exposure of water-soluble fractions of Hibernia crude oils at concentrations of 50-300ppb for 12-13 weeks were studied in the tissues of marine fish, Atlantic cod, *Gadus morhua L* (Khan *et al.*, 1987). Histopathological changes in oil-exposed fish included increased number of mucus-producing epithelial cells, capillary dilation, lamellar hyperplasia, and fusion of adjacent filaments in gills, microvascular formation in hepatocytes, delayed spermatogenesis with intratubular multinucleated giant cells, and an

increase of melanomacrophage centres in the spleen and kidney. Fatty degeneration was commonly found in the livers.

Paul *et al.* (2000) observed the occurrence of vibrosis in *M.dobsoni* exposed to water-accommodated fractions of crude oil. The prevalence of the coccidian parasite, *Goussia clupearum* and the fungus, *Ichthyophonus hoferi* was observed in adult Pacific herring, *Clupea pallasi* when exposed to Exxon Valdez oil. Recent laboratory studies have shown that exposure to crude oil resulted in dose-dependent expression of viral haemorrhagic septicaemia virus (VHSC) (Marty *et al.*, 1999).

# *Chapter* 2

## Effects Of Petroleum Hydrocarbons On Carbohydrate Metabolism

## 2A. INTRODUCTION

Petroleum hydrocarbons are known to influence physiological and biochemical state of aquatic organisms, by exhibiting marked changes in the activities of several enzymes of carbohydrate metabolism (Alkindi *et al.*, 1996; Omoregie, 1997, 2002).

Carbohydrates form the chief constituents of the energy sources for the vertebrates. Carbohydrate metabolism is broadly divided into two phases. Glycolysis is the process of degradation of glycogen or glucose to pyruvate and then to acetyl CoA to be utilized through Kreb's cycle. The purpose of TCA cycle is to produce as much energy as possible in the form of ATP molecules which is a main product in the biological oxidation (Robert *et al.*, 1998). Apart from this, there are also alternative degradative pathways like hexose monophosphate shunt. Besides, biosynthesis of glycogen occurs not only through glycogenesis from glucose but also from non-carbohydrate sources by gluconeogenesis. All these main pathways of carbohydrate metabolism are widely present in almost all tissues of the vertebrates. Functionally, the operation of such complex biosynthetic and biodegradative steps in carbohydrate metabolism certainly has a key role to play in supplying energy for cellular functions particularly under stress conditions like petroleum hydrocarbons induced toxicemia (Osmoregie, 1998).

The major carbohydrate prevalent in the liver of fish and in other vertebrates is glycogen which is synthesized either directly from glucose or from noncarbohydrate precursors and stored there. Anoxia and hypoxia are known to elevate carbohydrate consumption in petroleum hydrocarbon

environment (Val *et al.*, 1999; Duncan *et al.*, 2002). The level of tissue lactic acid is known to act as an index of anaerobiosis which might be beneficial to the fish to tolerate hypoxic conditions (Thoye, 1971; Wallice Luiz, 1998) under conditions like crude oil exposure.

It is well established that breakdown of glycogen to glucose-1-phosphate is regulated by phosphorylase enzyme (Robert *et al.*, 1998). Glycogen phosphorylase exists in two forms. Phosphorylase 'a' which is active favoring breakdown of glycogen to glucose-1-phosphate and phosphorylase 'b' which is inactive. The higher the phosphorylase 'a' activity, the more is the conversion of glycogen to glucose-1-phosphate. This enzyme is under the control of hormones like epinephrine and norepinephrine (Alkindi *et al.*, 1996). Estimation of phosphorylase 'a' (active form) and phosphorylase 'b' (inactive form) will give supporting evidence to evaluate the depletion of tissue glycogen content, the levels of which can serve as an indicator of stressful situations.

Lactate dehydrogenase (LDH) is a zinc containing enzyme. It is generally associated with cellular metabolic activities and plays a key role in energy metabolism. LDH acts as a pivotal enzyme between glycolytic pathway and TCA cycle. It catalyses the conversion of pyruvate into lactate under anaerobic conditions (Lehninger, 1993). A fish under stress preferentially meets its energy requirements through anaerobic oxidation. (Wallice Luiz, 1998). LDH can thus be used as an indicator in monitoring petroleum hydrocarbon induced toxicity in fish.

FAD-dependent succinate dehydrogenase facilitates interconversion of succinate and fumarate (Fukada, 1958) and it is mitochondrially localized. Malate dehydrogenase (MDH) is an $NAD^+$-dependent enzyme which converts malate to oxaloacetate but also plays a significant role in carbondioxide fixation and in gluconeogenesis (Robert *et al.*, 1998). Similarly oxidation of isocitrate to α-ketoglutarate is catalysed by $NADP^+$-dependent isocitrate dehydrogenase enzyme. Reduction in SDH activity is reported to be associated with a corresponding decrease in the other oxidative enzymes like malate dehydrogenase, isocitrate dehydrogenase and cytochrome-c-oxidase under various stress conditions (Dhalla *et al.*, 1971). These oxidative enzymes are of significance in the oxidative metabolism of the cell and any change in its activity is likely to disturb the harmony and co-ordination of the TCA cycle and other metabolic pathways.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are lipogenic enzymes concerned in HMP shunt pathway. Glucose-6-phosphate dehydrogenase is an NADP-dependent enzyme which catalyses the dehydrogenation of glucose-6-phosphate to 6-phosphogluconate. A second oxidative step is catalysed by 6-phosphogluconate dehydrogenase, which also requires $NADP^+$ as hydrogen acceptor. Decarboxylation follows with the formation of a keto pentose, ribulose-5-phosphate (Robert *et al.*, 1998).

Cytochrome-c-oxidase is a haemo-protein, widely distributed in animal tissues and acts as a terminal component of the respiratory chain in mitochondria. It transfers electrons to the final acceptor, oxygen. Being a terminal link in the electron transfer system, any change in its activity will

affect the synthesis of ATP molecules thereby influencing other cellular metabolic processes (Thomas *et al.*, 1997). It is well established that chemical compounds are known to form complexes with the oxidized form of cytochrome and this complex, after reduction by electrons passing down the chain from NADH, reacts with molecular oxygen (Wilkinson, 1980).

The toxic stress can only be understood by studying the physiological events associated with exposure to water-accommodated fractions of crude oil. Interestingly, the animals have the capacity to regulate and modulate the inherent diversions in their metabolism to meet the altered physiological or environmental conditions (Hoar, 1976). This is easily done to meet the energy demands under attenuated or imposed stress conditions to facilitate synthesis of extra energy to overcome such impeding situations.

In the light of above literature, some enzyme systems of carbohydrate metabolism in liver, gill, heart and muscle tissues were determined with reference to exposure to water- accommodated fractions of Bombay High crude oil for 21 days in *O.mossambicus*. The present investigation is aimed at understanding the possible metabolic regulatory phenomenon exhibited by the tissues under petroleum hydrocarbon toxicity stress as an attempt towards increased derivation of energy from these altered physiological pathway possibly to cope up with the increased energy demand augmented during toxic stress. Attempts were also made to investigate the biochemical adaptability of the animal to overcome the water-accommodated fractions of the crude oil induced toxicity by taking advantage of the metabolic diversion available at the time of toxicity.

Since the interactions are very common in the natural ecosystem polluted by petroleum hydrocarbons, investigations on carbohydrate metabolism were undertaken in fish intoxicated with water-accommodated fractions of Bombay High crude oil.

## 2B. MATERIALS AND METHODS

### 2B.1 Maintenance of Fish

*Oreochromis mossambicus* (Tilapia) of 15 ± 3 g were collected from the culture ponds of Rice Research Institute, Vyttila. Initially, they were kept in large tanks where a continuous and gentle flow of tap water was maintained. The tap water used had dissolved oxygen content of 7.8ppm, hardness – below detectable limits, pH 7.0 ± 0.37, temperature 26 ± 3° C and salinity 0 ppt. They were fed on a commercial diet *ad libitum* and were acclimated in tanks for a month before the experiment. For experimentation, the laboratory acclimatized fish were sorted into batches of eight each and kept in 180L polypropylene tanks containing tap water to maintain biomass theory (1 gm / 1L) (Muirhead Thomson, 1971; Holden, 1973). Daily water in the tanks was changed and aerated to prevent hypoxic conditions lest it may also influence toxicity. 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.

### 2B.2 Bioassay Method

The bioassay methods adopted in the present investigation were the same as that of Doudoroff *et al.* (1953). Fish of uniform size (15 ± 3 g)

were selected. For $LC_{50}$ determination (Finney, 1971) five concentrations of WAF (Water-accommodated fractions) of BHC (Bombay High crude) oil (10 to 30ppm) were selected. For each concentration, 12 fish were used, exposing 2 fish at a time in a 30 L polyprophylene tanks. This procedure was adopted throughout the investigation to maintain biomass theory. Each experiment was repeated six times at the selected water-accommodated fractions concentration, every time noting the number of fish killed at each concentration up to 96 hours. The tanks containing control and experimental fish were aerated throughout the experiment to prevent hypoxic condition if any (Khorram and Knight, 1977; Kabeer Ahammed Sahib, 1984; Siva Prasada Rao, 1984). The average mortality in each concentration was taken to determine the $LC_{50}$ by graphic method in which the prohibit mortality was plotted against log concentration of water-accommodated fractions by the procedure of Finney (1971). From this method, it was calculated that 1.5ppm was the sublethal water-accommodated fractions concentration for *O. mossambicus*. To know the effect of higher concentrations of water-accommodated fractions, apart from 1.5ppm concentration, two more concentrations such as 3.0ppm and 5.0 ppm of water-accommodated fractions were also selected for the present studies.

**2B.3 TOXICANT: Water-accommodated fractions (WAF) of Bombay High crude oil.**

Water-accommodated fractions (WAF) of Bombay High crude oil was prepared by the method of Anderson *et al.* (1974).

Water-accommodated fractions (WAF) of Bombay High crude (BHC) oil were prepared daily, by vortex mixing of oil with tap water in a ratio of 1 10 for 14h, in round perspex containers of 20 l capacity with bottom

outlets. After stirring, the mixture was allowed to settle for a period of 10 minutes and WAF was transferred for further separation into thoroughly cleaned separatory funnels of 2 l capacity for a period of 2 h and shaken well and the clear WAF was separated and transferred into clean beakers of 5 l capacity and this solution was treated as the 100% WAF The concentration of the accommodated oil was estimated in ppm basis after extraction of oil from the WAF using n-Hexane.

50 ml of the WAF was transferred into a hexane cleaned beaker of 100 ml capacity and acidified with 1 ml concentrated HCl to bring the pH below 2.This was extracted using 15 ml of n-Hexane (HPLC grade) by shaking in a separatory funnel for 2 minutes. The process was repeated twice. The combined n- Hexane extract was freed from residual water by treatment with anhydrous sodium sulphate (Sen Gupta *et al.*, 1980) and finally made upto 50 ml by the addition of n-Hexane. The fluorescence intensity of the n-Hexane extract was measured at wave lengths 310 nm (EX) and 360 nm (EM) using fluorescence spectrophotometer (Hitachi-Model F-3010) and the oil concentration in the 100% WAF was computed from the standard graph.

Calculated volumes of the WAF of the Bombay High crude oil was then added to the test media to get the required petroleum hydrocarbon concentrations.

### 2B.4 Experimental design for Water-accommodated fractions (WAF) exposure

For conducting biochemical and histological studies, *O.mossambicus* of 15 ± 3 g were taken in three separate tanks which contained desired

concentrations of WAF (1.5ppm, 3.0ppm and 5.0ppm respectively) obtained by mixing with tap water. Six replicates were kept for each experiment. The experimental animals were dosed for 3 weeks. Daily the contents in the tanks were replaced with the same concentrations of WAF so as to avoid any possible degradation or evaporation of constituents of WAF During the experimental period of 3 weeks the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. They were aerated throughout the experimental period to prevent hypoxic conditions. Any other factor likely to influence toxicity was nullified by maintaining suitable controls in tanks that contained no WAF

### 2B.5 Preparation of tissue samples and serum for the biochemical studies

After the experimental period (3 weeks) the fish were killed by pithing (by damaging the brain and severing the spinal cord between the head and the trunk region using a sharp needle) and the tissues viz. liver, gill, heart and muscle were removed from its body, wiped thoroughly using blotting paper to remove blood and other body fluids. Then they were washed in ice-cold 0.33 M sucrose and again blotted dry and desired amount of the tissues were weighed and used. Blood was drawn from the common cardinal vein in 1 ml plastic syringe and serum was separated from blood cells by centrifugation at 3000 rpm for 30 min and it was used for the various biochemical assays.

### 2B.6 METHODS USED FOR THE BIOCHEMICAL ANALYSIS

The following are the parameters studied under carbohydrate metabolism.

**a. Estimation of Total Carbohydrates**

The total carbohydrate was estimated by the method of Carrol *et al.* (1956).

**Reagents**

10% Trichloro acetic acid (TCA), anthrone reagent

**Procedure:**

0.5% homogenate of liver and 5% homogenate of muscle, heart and gill tissues were prepared in 10% Tri chloro acetic acid (TCA). The homogenates were centrifuged at 1000 g for 15 minutes. To 0.2 ml of the supernatant, 5 ml of anthrone reagent was added and boiled for 15 minutes. The tubes were cooled and the absorbance was read at 620 nm in a spectrophotometer using blank, which consists 10% TCA and anthrone reagent in the same proportion. The values were expressed as mg of glucose / g wet wt. of tissue.

**b. Estimation of Glycogen**

The tissue glycogen content was estimated by the method of Carrol *et al.* (1956).

**Reagents**

5% Trichloro acetic acid (TCA), 95% ethanol, standard glucose, anthrone reagent.

**Procedure**

10% of homogenates of muscle, heart and gill tissues and 0.5% homogenate of liver tissue were prepared in 5% TCA separately and centrifuged at 1000 g for 15 minutes. To one volume of the above supernatant 5 volumes of 95% ethanol was added and allowed to stand overnight in cold. After the precipitation was complete, the tubes were again centrifuged at 1000 g for 15 minutes. The supernatants were decanted and the residual fluid was allowed to drain off by invertedly keeping the tubes for about 10 minutes. The residue was dissolved in 1.0 ml of distilled water A reagent blank (1 ml of water) and a standard (1 ml of glucose solution containing 30 µg of glucose) were also prepared. 5 ml of anthrone reagent was added to each of the above tubes including blank and standard tubes. The tubes were kept for boiling for 15 minutes and then cooled to room temperature. The absorbance was read at 620 nm in a spectrophotometer using the blank. The values were expressed in mg / g wet wt. of tissue.

**c. Estimation of Blood Glucose**

Blood glucose was estimated by the method of Marks (1959) using o-Toluidine.

**Reagents**

1% o-Toluidine, 0.3 N NaOH, 0.15 M acetate buffer (pH 5.0), 0.02% peroxidase solution, Glucose oxidase, standard glucose, 5% $ZnSO_4.7H_2O$, Fermcozyme (a stable liquid preparation of glucose oxidase containing 750 units / ml).

**Procedure**

To 1.1 ml of 0.9% sodium chloride added 0.4 ml of 5% $ZnSO_4.7H_2O$ solution and 0.4 ml of 0.3 N NaOH. To this 0.1 ml of blood was added. Mixed well, centrifuged and separated the supernatant. Transferred 1 ml of this to a test tube and into two other tubes measured 1 ml of water as blank and 1 ml of standard glucose solution. Added 3 ml of glucose oxidase reagent to each at half minute intervals, mixed gently for not more than ten seconds, and read the absorbance at 625 nm. The values were expressed as mg glucose / dl.

**d. Estimation of Serum Lactate Dehydrogenase (LDH) (L-lactate:NAD Oxidoreductase; E.C.1.1.1.27)**

The serum lactate dehydrogenase was estimated by the method of Wroblewski *et al.* (1955).

**Reagents**

0.1 M phosphate buffer (pH 7.4), $NADH_2$ and Sodium pyruvate.

**Procedure**

Measured 2.7 ml of the phosphate buffer into a cuvette and added 0.1 ml of serum and 0.1 ml of $NADH_2$. Allowed to stand for 20 min at 37° C to reduce any keto acids already present in the serum. Then added 0.1 ml of sodium pyruvate. Read the absorbance for 5 min, at intervals of 15-30 sec at 340 nm in a spectrophotometer.

**e. Estimation of Tissue Lactate dehydrogenase**

Tissue Lactate dehydrogenase was estimated by the method of Bergmeyer and Bernt, 1974.

**Reagents**

50 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate, 0.18 mM NADH.

**Procedure**

The tissue was homogenized in 50 mM phosphate buffer (pH 7.5). The homogenate was centrifuged at 20,000 g for 30 min in a refrigerated centrifuge at 0°C. The supernatant obtained was used as the enzyme source. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 0.6 mM pyruvate, 0.18 mM NADH and the enzyme preparation. The reaction was initiated by the addition of enzyme and the assay was carried out at 30°C. The activity was determined from the rate of oxidation of NADH. The change in absorbance at 340 nm was measured in a spectrophotometer. A standard graph of NADH was prepared and the activity of the enzyme was expressed as mg of NADH oxidized per hour per gm protein in the sample. Protein content of the sample was estimated by following the procedure of Lowry *et al.* (1951).

**f. Estimation of Lactic acid**

The tissue lactic acid content was estimated by the method of Barker and Summerson, 1941 as modified by Huckabee, 1961.

**Reagents**

10% Trichloro acetic acid (TCA), 20% copper sulphate, calcium hydroxide, analar sulphuric acid, p-hydroxy diphenyl.

**Procedure**

After isolating the tissues, 10% homogenates of muscle, heart and gill tissues and 5% homogenate of liver tissue were prepared in 10% cold TCA and centrifuged at 1000 g for 15 minutes. To 1.0 ml of supernatant, 1.0 ml of 20% copper sulphate solution was added. The contents were mixed and the volume was made up to 10.0 ml with distilled water. To it 1 gram of powdered calcium hydroxide was added and contents were mixed vigorously till the dissolution of calcium hydroxide. The tubes were kept aside for 1 hour at room temperature giving intermittent shaking and later centrifuged at 3000g for 10 minutes. To 1.0 ml of the supernatant, 0.05 ml of 4% copper sulphate solution and 6.0 ml of analar sulphuric acid were added. The contents were mixed well by lateral shaking and then boiled in a water bath for 6 minutes. After cooling 0.1 ml of p-hydroxy diphenyl was added to the solution and kept at room temperature for 30 minutes. Again, the contents were boiled in a water bath for 1 minute. After cooling, the absorbance was read against a reagent blank at 560 nm in a spectrophotometer. The blank received the same treatment as that of the samples, except that distilled water replaced the homogenate. The values were expressed as mg lactic acid / g wet wt. of tissue.

**g. Estimation of Pyruvate**

Pyruvate was estimated by the method of Friedemann and Haugen, 1943.

**Reagents**

10% Trichloro acetic acid (TCA), 0.1% .2, 4 -Dinitrophenyl hydrazine (DNPH), 2.5 N NaOH

**Procedure**

10% homogenates of muscle, heart and gill tissues and 5% homogenate of liver tissue were prepared in 10% TCA and centrifuged at 1000 g for 15 minutes. To 2.0 ml of supernatant, 0.5 ml of 0.1% 2, 4-Dinitrophenyl hydrazine (DNPH) was added and the tubes were kept for 5 minutes at room temperature and 3.0 ml of 2.5 N NaOH solution was added. After 10 minutes the absorbance was read at 540 nm in a spectrophotometer against a reagent blank. The blank consisted of 2.0 ml of 10% TCA, 0.5 ml of 0.1% 2, 4 -DNPH and 3.0 ml of 2.5 N NaOH solution. The standard graph was prepared by using sodium pyruvate. The values were expressed as μ moles of pyruvate / g wet wt. of tissue.

**h. Estimation of Glycogen Phosphorylase (α-1, 4-glycogen: Orthophosphate glycosyl-transferase (E.C. 2.4.1.1)**

Phosphorylase activity was assayed by the method of Cori *et al.* (1955) in the direction of glycogen synthesis by estimating the inorganic phosphate formed from glucose-1- phosphate.

**Reagents**

0.1 M sodium fluoride, 0.037 M ethylenediamine tetracetate (EDTA) (pH 6.5), 0.03 M cysteine, 0.015 M β-glycerophosphate (pH 6.5), 2%

glycogen, 0.016 M glucose-1-phosphate, 0.04 M adenosine-5-monophosphate (AMP), ammonium molybdate, 1-amino-2-napthol-4-sulphonic acid (ANSA).

**Procedure**

5% homogenate of muscle, heart, gill and liver tissues were prepared in aqueous medium containing 0.1 M sodium fluoride and 0.037 M ethylenediamine tetracetate (EDTA) of pH 6.5 as suggested by Guillory and Mommaerts (1962) to avoid enzymatic interconversion of the two phosphorylases. The homogenates were centrifuged at 1000 g for 15 minutes. The supernatants were diluted four fold with cysteine (0.03 M) - β-glycerophosphate (0.015 M) buffer of pH 6.5. 0.4 ml of the dilute supernatant was added to 0.2 ml of 2% glycogen and incubated for 20 minutes at 35°C. The reaction was initiated by adding 0.2 ml of glucose-1-phosphate (0.016M) to one of the tubes and to the other 0.2 ml of mixture of 0.016 M glucose-1-phosphate and 0.04 M adenosine-5-monophosphate (AMP) for estimating the active phosphorylase (a) and total phosphorylase (ab) respectively After incubating for 15 minutes for total phosphorylase and 30 minutes for active phosphorylase, 0.5 ml of aliquots were pipetted and added in another tube containing 5 ml of dilute sulphuric acid (10 ml of 5N $H_2SO_4$+ 690 ml of distilled water) to stop the enzymatic action.

The inorganic phosphate formed was estimated by the method of Fiske and Subba Row (1925). The absorbance of the blue colour was read within 5 minutes at 660 nm in a spectrophotometer against zero time controls.

## Estimation of Inorganic Phosphate

To 1.0 ml of the filtrate, 1.0 ml of ammonium molybdate solution was added and mixed well and 0.4 ml of 1-amino-2-napthol-4-sulphonic acid (ANSA) reagent was added. The contents were made upto 10 ml with distilled water and allowed to stand for 5 minute. The absorbance of the blue colour was measured at 720 nm in spectrophotometer against a blank. The blank contains 1.0 ml of distilled water, 1.0 ml of ammonium molybdate and 0.4 ml of ANSA, made upto 10 ml with distilled water.

## Enzymes of HMP shunt pathway (Pentose Phosphate Pathway)

### i. Estimation of Glucose-6-phosphate dehydrogenase (D-Glucose-6-phosphate: $NADP^+$ Oxidoreductase) (E.C. 1.1.1.49)

Glucose-6-phosphate dehydrogenase activity in tissues was estimated by the method of Kornberg and Horecker, 1955.

#### Reagents

0.33m sucrose, 0.04M glycyl glycine buffer (pH 7.5), 0.02M glucose-6-phosphate, $1.5 \times 10^{-3}$ $NADP^+$ 0.1M Magnesium chloride.

#### Procedure

The chilled tissue were homogenized with 3 volumes of 0.33 M sucrose solution and centrifuged at 1000 g for 10 minutes. The supernatant was used as the enzyme source for the assay. To 0.1 ml of the substrate (0.02M glucose-6-phosphate) in a quartz cell, 0.1 ml of $1.5 \times 10^{-3}$ $NADP^+$ 0.25 ml of buffer and 0.2 ml of 0.1 M $MgCl_2$ were added. To this 0.05 ml of enzyme

was added and the absorbance was read immediately at 340nm at 20 second intervals. A unit of enzyme is defined as that amount of enzyme which causes an increase in optical density of 1.0 per minute under the above conditions of assay. Specific activity is expressed as units per mg of protein.

**j. Estimation of 6-phosphogluconate dehydrogenase (E.C. 1.1.1.43)**

The procedure was same as glucose-6-phosphate dehydrogenase except that 6-phosphogluconate was used as substrate instead of glucose-6-phosphate.

**k. Estimation of Succinate Dehydrogenase (SDH) (Succinate: Acceptor Oxidoreductase: E.C. 1.3.99.1)**

Succinate dehydrogenase activity was estimated by the method of Nachlas *et al.* (1960).

**Reagents**

0.33 M sucrose, 40 µM sodium succinate, 100 µM of potassium phosphate buffer (pH 7.0), 4 µM INT (2-p-idodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride), Toluene.

**Procedure**

10% homogenate of the gill tissue and 5% homogenates of heart, muscle and liver tissues were prepared in cold 0.25 M sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatants were used for the assay. The reaction mixture of 2.0 ml contained 40 µ moles of sodium

succinate, 100 µ moles of potassium phosphate (pH 7.0) buffer, 4 µ moles of INT (2-p-iodophenyl)-3-(p -nitrophenyl)-5-phenyl tetrazolium chloride) and 0.5 ml of supernatant. The contents were incubated for 30 minutes at 37°C and the reaction was arrested by adding 5.0 ml of glacial acetic acid. The iodoformazan formed was extracted overnight in 5.0 ml of toluene at 5°C. The absorbance of the colour was measured in a spectrophotometer at 495 nm against a blank. Toluene is used as blank. The enzyme activity is expressed as µ moles of formazan formed / mg protein / h.

**I. Estimation of Malate Dehydrogenase (MDH) (L-malate-NAD$^+$ Oxidoreductase; E.C.1.1.1.37)**

Malate dehydrogenase activity was estimated by the method of Nachlas *et al.* (1960).

**Reagents**

40 µM of sodium malate, 100 µM potassium phosphate buffer (pH 7.0), 0.1 µM of nicotinamide adenine dinucleotide (NAD), 4 µM of INT (2-p-idodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride).

**Procedure**

The procedure adopted for initial tissue processing was the same as done for SDH. The reaction mixture of 2.0 ml contained; 40 µ moles of sodium malate, 100 µ moles of potassium phosphate buffer (pH 7.0), 0.1 µ mole of nicotinamide adenine dinucleotide (NAD), 4 µ moles of INT and 0.5 ml of the homogenate supernatant. The other steps followed were the same

as those described for SDH. The enzyme activity is expressed as μ moles of formazan formed / mg protein / h.

**Enzymes of Citric acid cycle**

**m. Estimation of Isocitrate dehydrogenase (Isocitrate: $NAD^+$ Oxidoreductase; E.C.1.1.1.41)**

Isocitrate dehydrogenase activity was estimated by the method of Kornberg (1951).

**Reagents**

0.5 M $KH_2PO_4$-$K_2HPO_4$ (pH 7.0), 0.1 M Magnesium Chloride, 0.025 M adenosine-5-phosphate, 0.05 M $NAD^+$ 0.005 M dl-isocitrate.

**Procedure**

The reaction mixture contained phosphate buffer (2 ml), magnesium chloride (1 ml), adenosine-5-phosphate (0.2 ml), $NAD^+$ (0.2 ml), the enzyme solution (0.1 ml), dl-isocitrate (1ml) and water (1 ml). The assay was run at room temperature. A control containing all reaction components except isocitrate was incubated. The increase in optical density at 340nm resulting from the reduction of $NAD^+$ was observed at 30 sec intervals for 3 min.

**Enzyme of electron transport chain**

**n. Estimation of Cytochrome-c-oxidase (Ferrocytochrome c: Oxygen Oxidoreductase; E.C. 1.9.3.1)**

Cytochrome-c-oxidase activity was estimated by the method of Oda *et al.* (1958).

**Reagents**

0.1 M phosphate buffer (pH 7.6), 0.2 M p-phenylenediamine, 0.2% Neotetrazolium chloride, $10^{-4}$ M cytochrome-c, 1:1 ether: acetone mixture.

**Procedure**

10% homogenate of muscle, gill, heart and liver tissues were prepared in 0.1 M cold phosphate buffer of pH 7.6, and centrifuged at 1000 g for 15 minutes. The supernatants were used for the assay. The reaction mixture of 0.8 ml contained: 0.2 ml of 0.2 M p-phenylenediamine, 0.2 ml of 0.2% Neotetrazolium chloride, 0.2 ml of $10^{-4}$ M cytochrome-c and 0.2 ml of supernatant as enzyme source. The contents were incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 0.4 ml of 1N $H_2SO_4$. The yellowish-brown precipitate is the oxidized product of p-phenylenediamine, which was extracted overnight in 5.0 ml of 1:1 ether: acetone mixture. Its absorbance was measured in a spectrophotometer at 520 nm against a blank. The ether: acetone (1:1) mixture served as blank. The enzyme activity was expressed in µg of diformazan formed / mg protein / h.

**2C. RESULTS**

Effect of 1.5ppm, 3.0ppm and 5.0ppm concentrations of WAF exposure for three weeks on the total carbohydrates, glycogen, blood glucose, serum lactate dehydrogenase, tissue lactate dehydrogenase, lactic acid, pyruvate, phosphorylase 'a', phosphorylase 'b', glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase and cytochrome-c-oxidase on

liver, gill, heart and muscle tissues of *O.mossambicus* are given in tables 2.1 to 2.15 and in figures 2.1 to 2.13. Results were statistically analyzed by ANOVA (Analysis of Variance) followed by LSD (Least significance difference) analysis (Zar, 1996).

**Table 2.1 Effect of different concentrations of WAF exposure on the total carbohydrate content in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 46.35 ± 0.78 | 4.52 ± 0.55 | 11.25 ± 0.37 | 17.00 ± 0.09 |
| 1.5ppm | 43.24 ± 1.07 | 3.89 ± 0.57 | 9.04 ± 0.06 | 15.27 ± 0.36 |
| 3.0ppm | 40.12 ± 1.57 | 1.05 ± 0.06 | 5.83 ± 0.12 | 10.68 ± 1.13 |
| 5.0ppm | 36.31 ± 2.11 | 0.546 ± 0.03 | 2.02 ± 0.06 | 8.54 ± 0.32 |

⇒ Values are expressed as mg / g wet wt. of tissue.

⇒ Average of six values in each groups ± SD.

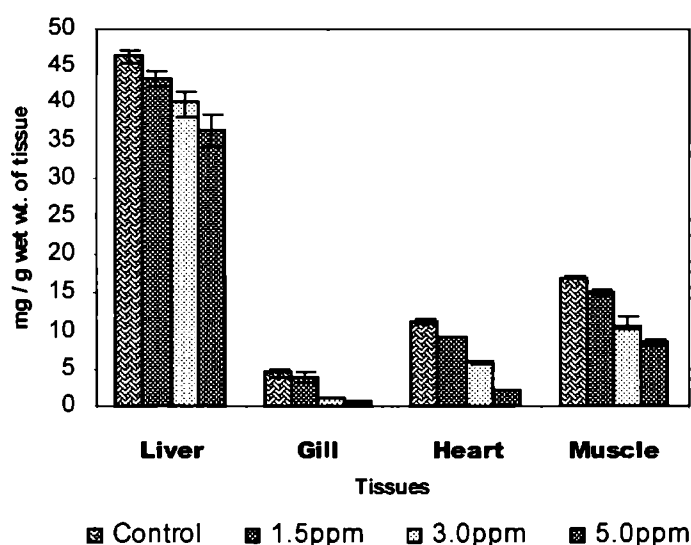



**Figure 2.1 Levels of total carbohydrates in the various tissues exposed to different concentrations of WAF**

**Table 2.1a Two-Factor ANOVA for total carbohydrate**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 154.2494 | 3 | 51.41645 | 36.36387 | 2.33E-05 | 3.862539 |
| Between tissues | 3656.355 | 3 | 1218.785 | 861.9759 | 2.2E-11 | 3.862539 |
| Error | 12.7549 | 9 | 1.413943 | | | |
| Total | 3923.33 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA revealed an overall significant decrease (P<0.001) (Table 2.1 a) in total carbohydrate between different concentrations of WAF treated group with the control group. Further comparison by LSD analysis (Table 2.6 b) revealed that no significant difference was observed between control and 1.5ppm. All other concentration comparisons gave significant difference. LSD at 5% level was 1.9018. 5ppm concentration of WAF treatment gave significantly higher values when compared to other concentrations. Subsequent comparison by LSD analysis (Table 2.6 c) revealed a significant difference (P<0.001) between tissues.

**Table 2.2 Effect of different concentrations of WAF exposure on the glycogen content in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 22.98 ± 0.58 | 3.39 ± 0.23 | 9.24 ± 0.84 | 10.22 ± 0.77 |
| 1.5ppm | 17.32 ± 0.34 | 1.85 ± 0.21 | 6.31 ± 0.74 | 7.25 ± 0.68 |
| 3.0ppm | 15.63 ± 0.60 | 0.84 ± 0.05 | 4.16 ± 0.55 | 5.68 ± 0.51 |
| 5.0ppm | 12.12 ± 0.23 | 0.49 ± 0.04 | 2.47 ± 0.38 | 3.24 ± 0.21 |

⇒ Values are expressed as mg / g wet wt. of tissue.

⇒ Average of six values in each groups ± SD.

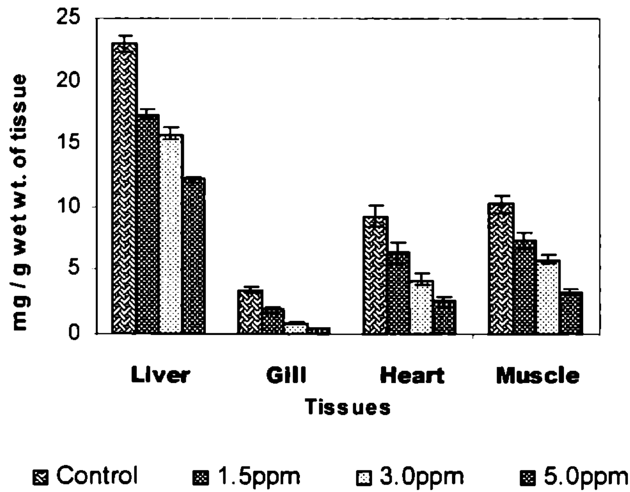



**Figure 2.2 Levels of Glycogen in the various tissues exposed to different concentrations of WAF**

**Table 2.2a Two-Factor ANOVA for Glycogen**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 101.3841 | 3 | 33.79469 | 18.41845 | 0.000351 | 3.862539 |
| Between tissues | 517.102 | 3 | 172.3673 | 93.94193 | 4.12E-07 | 3.862539 |
| Error | 16.51346 | 9 | 1.834828 | | | |
| Total | 634.9995 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 2.2 a ) revealed a significant decrease (P<0.05) in glycogen content between WAF treated groups with the control. Subsequent comparison by LSD analysis (Table 2.6 b) showed that there was a significant decrease between control and 1.5 ppm, 3.0 ppm, 5.0 ppm treated groups. Comparison between the different concentrations revealed that there was no significant difference between 3.0 ppm and 5.0 ppm treated groups and also between 3.0 ppm and 1.5 ppm treated groups. Whereas comparison between 5.0 ppm and 1.5 ppm WAF treated group showed a significant

difference. Further comparison between tissues revealed (Table 2.6 c) a significant variation (P<0.001) except between muscle and heart tissues.

**Table 2.3 Effect of different concentrations of WAF exposure on the Blood Glucose level in *O. mossambicus***

| GROUP | Concentrations of WAF exposure | | |
|---|---|---|---|
| Control | 1.5ppm | 3.0ppm | 5.0ppm |
| 42.85 ± 3.45 | 48.85 ± 1.41 | 53.35 ± 0.64 | 61.72 ± 3.04 |

⇒ Values are expressed in mg / 100 ml of blood.
⇒ Average of six values in each groups ± SD.

**Table 2.3a One way ANOVA for Blood Glucose**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 1151.77 | 3 | 383.9233 | 65.14728 | 1.68E-10 | 3.098393 |
| Within concentrations | 117.8632 | 20 | 5.89316 | | | |
| Total | 1269.633 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

One way ANOVA showed significant increase (P<0.001) (Table 2.3 a) in the blood glucose level between control and different concentrations of WAF treated groups. From subsequent LSD analysis revealed that 5.0ppm concentration of WAF enhanced blood glucose level significantly (P<0.001) than other two concentrations. LSD level at 5% level was 2.9292.

**Table 2.4 Effect of different concentrations of WAF exposure on Lactate dehydrogenase (LDH) activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 61.44 ± 3.01 | 18.86 ± 1.72 | 22.98 ± 2.63 | 36.68 ± 3.15 |
| 1.5ppm | 63.85 ± 2.42 | 19.73 ± 1.97 | 26.61 ± 3.25 | 36.17 ± 1.85 |
| 3.0ppm | 71.61 ± 1.73 | 25.74 ± 2.04 | 38.59 ± 1.82 | 41.22 ± 2.14 |
| 5.0ppm | 82.95 ± 2.85 | 31.68 ± 1.85 | 40.26 ± 3.16 | 45.58 ± 1.76 |

⇒ Values are expressed as μ moles of NADH oxidized / h / gm protein.
⇒ Average of six values in each groups ± SD.

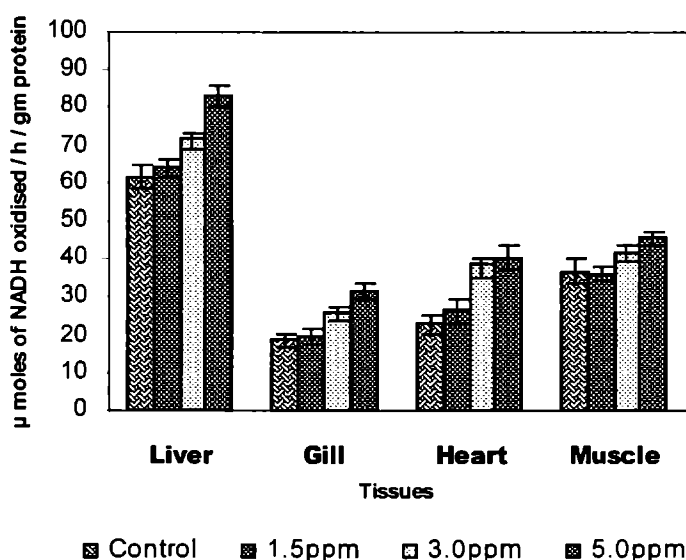



**Figure 2.3 Activity of LDH in the various tissues exposed to different concentrations of WAF**

**Table 2.4 a Two-Factor ANOVA for LDH activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 594.1343 | 3 | 198.0448 | 24.0156 | 0.000125 | 3.862539 |
| Between tissues | 4827.875 | 3 | 1609.292 | 195.1483 | 1.66E-08 | 3.862539 |
| Error | 74.21856 | 9 | 8.246506 | | | |
| Total | 5496.227 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 2.4 a) on tissue LDH activity studies revealed an overall significant increase ($P<0.001$) between different concentrations of WAF treatment with control group. Comparison by LSD analysis (Table 2.6 b) revealed that there was no significant difference between control and 1.5ppm treated group. Whereas there was a significant difference between control and 3.0ppm as well as control and 5.0ppm treated

groups. LSD value at 5% level was 4.5931. Similarly there was a significant difference (P<0.001) between tissues (Table 2.6 c).

**Table 2.5 Effect of different concentrations of WAF exposure on the activity of Serum Lactate dehydrogenase (LDH) in *O. mossambicus*.**

| Control | Concentrations of WAF exposure | | |
|---|---|---|---|
| | 1.5ppm | 3.0ppm | 5.0ppm |
| 114.85 ± 2.75 | 173.63 ± 4.82 | 190.68 ± 7.37 | 232.58 ± 5.61 |

⇒ Values are expressed as Units / L.
⇒ Average of six values in each groups ± SD.

**Table 2.5a One way ANOVA for Serum LDH**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 42877.29 | 3 | 14292.43 | 391.2439 | 6.4E-18 | 3.098393 |
| Within concentrations | 730.6147 | 20 | 36.53074 | | | |
| Total | 43607.9 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Serum Lactate dehydrogenase activity showed an overall significant increase (P<0.001) in WAF treated groups when compared to control (Table 2.5a). The maximum increase in LDH activity was observed in fish exposed to 5.0ppm which was followed by 3.0ppm and 1.5ppm treated groups. LSD value at 5% level was 7.293. Comparison between the concentrations also revealed a significant difference (P<0.001).

**Table 2.6 Effect of different concentrations of WAF exposure on the Lactic acid content in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 1.85 ± 0.17 | 1.13 ± 0.14 | 1.01 ± 0.02 | 3.03 ± 0.22 |
| 1.5ppm | 2.22 ± 0.28 | 1.64 ± 0.21 | 1.52 ± 0.19 | 3.81 ± 0.23 |
| 3.0ppm | 3.44 ± 0.34 | 1.93 ± 0.17 | 1.88 ± 0.41 | 4.53 ± 0.38 |
| 5.0ppm | 4.95 ± 0.25 | 2.72 ± 0.27 | 2.89 ± 0.44 | 5.17 ± 0.34 |

⇒ Values are expressed as mg / g wet wt. of tissue.

⇒ Average of six values in each groups ± SD

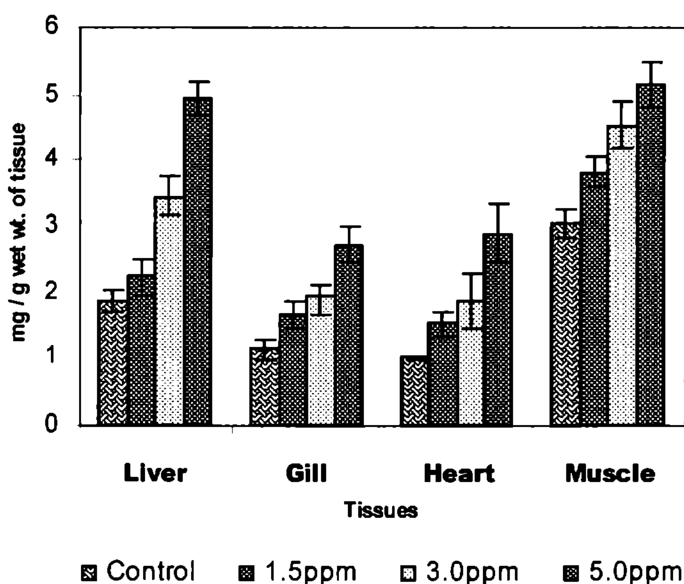



**Figure 2.4 Levels of Lactic acid in the various tissues exposed to different concentrations of WAF**

**Table 2.6a Two-Factor ANOVA for Lactic Acid**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 10.51955 | 3 | 3.506517 | 27.96141 | 6.81E-05 | 3.862539 |
| Between tissues | 14.8275 | 3 | 4.9425 | 39.41213 | 1.67E-05 | 3.862539 |
| Error | 1.12865 | 9 | 0.125409 | | | |
| Total | 26.4757 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two way ANOVA revealed an overall significant increase in lactic acid (P<0.001) between different concentrations of WAF with the control group (Table 2.6a). Comparison by LSD analysis (Table 2.6 b) revealed that all the concentrations caused significant increase (P<0.001) in lactic acid when compared to control except 1.5ppm treated group. LSD value at 5% level was 0.5664. Comparison between tissues (Table 2.6c) also revealed a significant difference (P<0.001).Muscle tissue showed maximum lactic acid level than all other tissues. However, there was no significant difference between cardiac and gill tissues.

**Table 2.6 b Results of LSD analysis for different WAF concentrations.**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Total Carbohydrates | Glycogen | LDH | Lactic acid |
| Control Vs 1.5ppm | NS | P < 0.05 | NS | NS |
| Control Vs 3.0ppm | P < 0.001 | P < 0.01 | P < 0.05 | P < 0.05 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 1.5ppm | P < 0.05 | NS | P < 0.05 | P < 0.05 |
| 5.0ppm Vs 1.5ppm | P < 0.001 | P < 0.01 | P < 0.001 | P < 0.05 |
| 5.0ppm Vs 3.0ppm | P < 0.01 | NS | P < 0.01 | P < 0.01 |

NS-Not significant

**Table 2.6 c Results of LSD analysis for different tissues.**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Total Carbohydrates | Glycogen | LDH | Lactic acid |
| Liver Vs Gill | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.05 |
| Liver Vs Heart | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.05 |
| Liver Vs Muscle | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.05 |
| Heart Vs Gill | P < 0.05 | P < 0.05 | P < 0.01 | NS |
| Muscle Vs Gill | P < 0.001 | P < 0.05 | P < 0.001 | P < 0.001 |
| Muscle Vs Heart | P < 0.05 | NS | P < 0.01 | P < 0.001 |

NS-Not significant
LDH-Lactate dehydrogenase

**Table 2.7 Effect of different concentrations of WAF exposure on the pyruvate level in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 7.85 ± 0.45 | 3.11 ± 0.39 | 4.42 ± 0.53 | 5.42 ± 0.35 |
| 1.5ppm | 6.74 ± 0.25 | 2.43 ± 0.41 | 3.61 ± 0.41 | 4.07 ± 0.22 |
| 3.0ppm | 3.89 ± 0.54 | 1.05 ± 0.12 | 2.03 ± 0.13 | 2.49 ± 0.43 |
| 5.0ppm | 2.74 ± 0.37 | 0.92 ± 0.15 | 1.63 ± 0.29 | 1.86 ± 0.24 |

⇒ Values are expressed as μ moles / g wet wt. of tissue.

⇒ Average of six values in each groups ± SD.

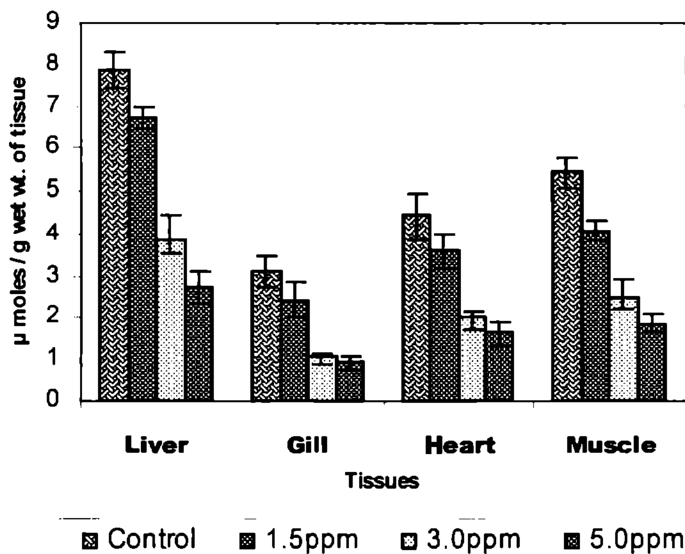



**Figure 2.5 Levels of pyruvate in the various tissues exposed to different concentrations of WAF**

**Table 2.7a Two-Factor ANOVA for Pyruvate**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 30.28493 | 3 | 10.09498 | 28.79228 | 6.05E-05 | 3.862539 |
| Between tissues | 24.71332 | 3 | 8.237775 | 23.49529 | 0.000136 | 3.862539 |
| Error | 3.155525 | 9 | 0.350614 | | | |
| Total | 58.15377 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA revealed that there was an overall significant decrease in pyruvate (P<0.001) (Table 2.7a) between control and different WAF concentrations. Subsequent comparison by LSD analysis revealed (Table 2.10 b) that between concentrations, there was a significant decrease (P<0.001). Different concentrations of WAF treated groups were compared with the control showed significant difference. However there was no significant difference between 5.0ppm and 3.0ppm treated groups. Comparison between tissues by LSD analysis (Table2.10c) also revealed that there was a significant difference. Hepatic tissues showed maximum pyruvate content whereas as gill tissue showed minimum. However there was no significant difference between cardiac and muscle tissues.

**Table 2.8 Effect of different concentrations of WAF exposure on the level of Phosphorylase 'a' activity in different tissues of *O.mossambicus.***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 5.27 ± 0.55 | 1.42 ± 0.35 | 3.84 ± 0.14 | 2.75 ± 0.39 |
| 1.5ppm | 8.14 ± 0.16 | 3.02 ± 0.43 | 6.02 ± 0.16 | 4.43 ± 0.31 |
| 3.0ppm | 10.67 ± 0.47 | 5.63 ± 0.26 | 8.14 ± 0.49 | 6.24 ± 0.23 |
| 5.0ppm | 11.82 ± 0.34 | 7.68 ± 0.29 | 9.12 ± 0.28 | 8.23 ± 0.54 |

⇒ Values are expressed as μ moles of inorganic phosphate formed / mg protein / h.

⇒ Average of six values in each groups ± SD.

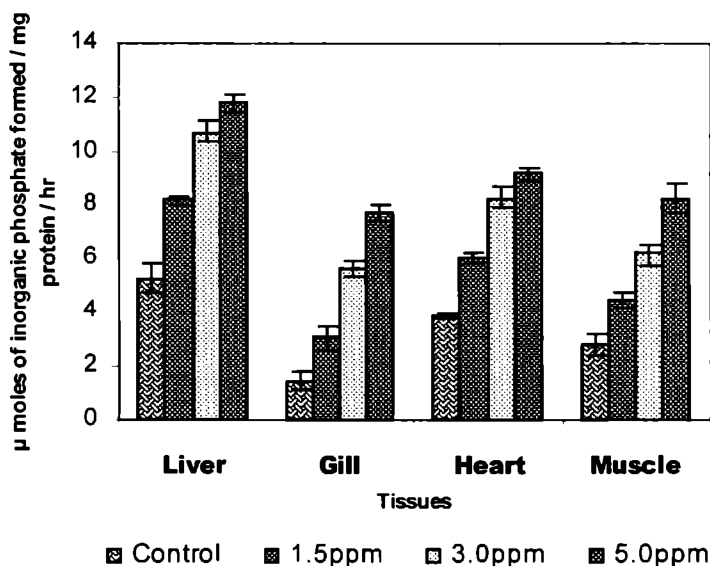



**Figure 2.6 Activity of Glycogen Phosphorylase 'a' in the various tissues exposed to different concentrations of WAF**

**Table 2.8a Two -Factor ANOVA for Phosphorylase 'a'**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 80.01782 | 3 | 26.67261 | 145.6967 | 6.04E-08 | 3.862539 |
| Between tissues | 46.40632 | 3 | 15.46877 | 84.49676 | 6.53E-07 | 3.862539 |
| Error | 1.647625 | 9 | 0.183069 | | | |
| Total | 128.0718 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 2.8a) revealed an overall significant increase in phosphorylase 'a' activity ($P<0.001$) between control and different concentrations of WAF treated groups. Further comparison by LSD (Table 2.10 b) between 1.5ppm, 3.0ppm and 5.0ppm with the control showed a significant change. Comparison between tissues by LSD analysis (Table 2.10 c) also showed a significant difference. Hepatic tissue exhibits significantly higher value followed by cardiac, muscle and gill tissues.

**Table 2.9 Effect of different concentrations of WAF exposure on the level of Phosphorylase 'b' activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 3.25 ± 0.31 | 1.52 ± 0.32 | 2.31 ± 0.16 | 2.13 ± 0.11 |
| 1.5ppm | 2.86 ± 0.22 | 0.97 ± 0.10 | 1.34 ± 0.23 | 1.59 ± 0.18 |
| 3.0ppm | 1.85 ± 0.18 | 0.45 ± 0.11 | 0.74 ± 0.15 | 0.68 ± 0.12 |
| 5.0ppm | 1.07 ± 0.11 | 0.31 ± 0.07 | 0.51 ± 0.12 | 0.45 ± 0.11 |

⇒ Values are expressed as µ moles of inorganic phosphate formed / mg protein / h.
⇒ Average of six values in each groups ± SD.

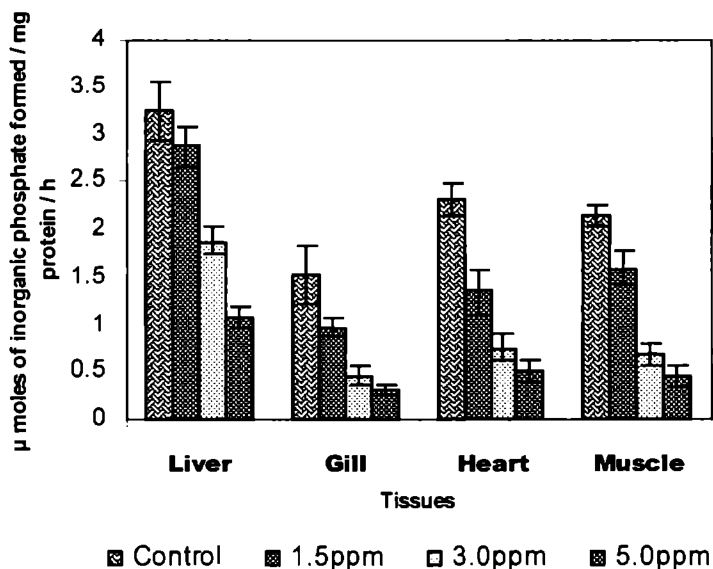



**Figure 2.7 Activity of Glycogen Phosphorylase 'b' in the various tissues exposed to different concentrations of WAF**

**Table 2.9 a Two-Factor ANOVA for Phosphorylase 'b' activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 7.126369 | 3 | 2.375456 | 43.33401 | 1.13E-05 | 3.862539 |
| Between tissues | 4.576419 | 3 | 1.525473 | 27.82828 | 6.94E-05 | 3.862539 |
| Error | 0.493356 | 9 | 0.054817 | | | |
| Total | 12.19614 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two way ANOVA (Table 2.9 a) revealed an overall significant decrease in phosphorylase 'b' activity between control group with that of different concentrations of WAF treated groups. Subsequent comparison by LSD (Table 2.10 b) between control and 1.5ppm, 3.0ppm and 5.0ppm WAF treated groups revealed a significant difference. Comparison between concentrations showed a significant difference except between 5.0ppm and 3.0ppm treated groups. Comparison between tissues by LSD analysis (Table 2.10c) also revealed a significant difference except between heart and gill; muscle and gill and also between muscle and heart.

**Table 2.10 Effect of different concentrations of WAF exposure on the Glucose-6-phosphate dehydrogenase (G-6-PD) activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 32.46 ± 1.23 | 10.61 ± 0.94 | 17.31 ± 0.98 | 16.37 ± 1.33 |
| 1.5ppm | 36.79 ± 0.93 | 11.25 ± 1.22 | 20.42 ± 1.07 | 19.48 ± 1.75 |
| 3.0ppm | 38.39 ± 1.17 | 15.25 ± 0.72 | 23.35 ± 0.95 | 22.15 ± 1.47 |
| 5.0ppm | 46.55 ± 1.82 | 18.23 ± 0.97 | 25.43 ± 1.11 | 24.69 ± 1.25 |

⇒ Values are expressed in units / mg protein.

⇒ Average of six values in each groups ± SD.

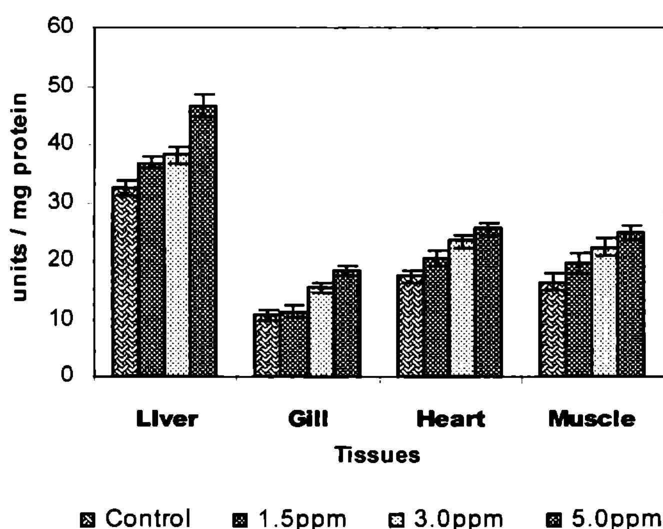



**Figure 2.8 Activity of G-6-PD in the various tissues exposed to different concentrations of WAF**

**Table 2.10 a Two-Factor ANOVA for G-6-PD activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 198.9131 | 3 | 66.30437 | 30.63185 | 4.71E-05 | 3.862539 |
| Between tissues | 1324.896 | 3 | 441.6321 | 204.0289 | 1.36E-08 | 3.862539 |
| Error | 19.48101 | 9 | 2.164556 | | | |
| Total | 1543.29 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

Two-Factor ANOVA (Table 2.10a) revealed an overall significant increase (P<0.001) in glucose-6-phosphate dehydrogenase activity between control and different concentrations of WAF treated groups. Subsequent comparison by LSD analysis (Table 2.10b) revealed that there was no significant difference between control and 1.5ppm treated group, whereas other two concentrations gave significant increase when compared to control. Comparison between tissues as obtained by LSD analysis (Table 2.10c) revealed that there was a significant difference with the exception of muscle and heart.

**Table 2.10 b Results of LSD analysis for different WAF concentrations.**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Pyruvate | Phosphorylase 'a' | Phosphorylase 'b' | G6PD |
| Control Vs 1.5ppm | NS | P < 0.05 | P < 0.05 | NS |
| Control Vs 3.0ppm | P < 0.05 | P < 0.001 | P < 0.001 | P < 0.01 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.05 |
| 3.0ppm Vs 1.5ppm | P < 0.01 | P < 0.05 | P < 0.05 | NS |
| 5.0ppm Vs 1.5ppm | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.01 |
| 5.0ppm Vs 3.0ppm | NS | P < 0.01 | NS | P < 0.05 |

NS-Not significant

**Table 2.10 c Results of LSD analysis for different tissues.**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Pyruvate | Phosphorylase 'a' | Phosphorylase 'b' | G6PD |
| Liver Vs Gill | P < 0.001 | P < 0.001 | P < 0.05 | P < 0.001 |
| Liver Vs Heart | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.001 |
| Liver Vs Muscle | P < 0.01 | P < 0.001 | P < 0.05 | P < 0.001 |
| Heart Vs Gill | P < 0.01 | P < 0.05 | NS | P < 0.001 |
| Muscle Vs Gill | P < 0.01 | P < 0.01 | NS | P < 0.01 |
| Muscle Vs Heart | NS | P < 0.01 | NS | NS |

NS-Not significant

G6PD-Glucose-6-phosphate dehydrogenase

**Table 2.11 Effect of different concentrations of WAF exposure on the 6-phospho gluconate dehydrogenase (6-PGAD) activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 3.96 ± 0.53 | 1.35 ± 0.11 | 2.45 ± 0.10 | 1.22 ± 0.07 |
| 1.5ppm | 4.11 ± 0.24 | 1.85 ± 0.09 | 2.92 ± 0.08 | 1.79 ± 0.05 |
| 3.0ppm | 4.88 ± 0.47 | 2.36 ± 0.14 | 3.67 ± 0.24 | 2.64 ± 0.24 |
| 5.0ppm | 5.86 ± 0.51 | 3.63 ± 0.22 | 4.01 ± 0.17 | 3.75 ± 0.35 |

⇒ Values are expressed in units / mg protein.

⇒ Average of six values in each groups ± SD.

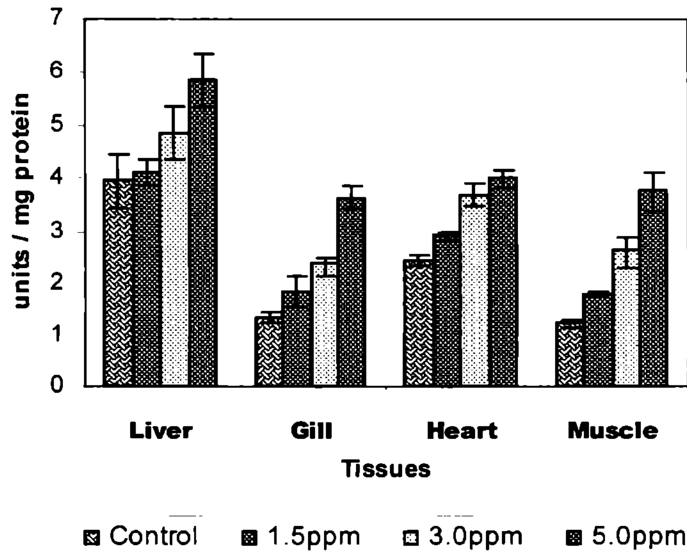



**Figure 2.9 Activity of 6-PGAD in the various tissues exposed to different concentrations of WAF**

**Table 2.11 a Two-Factor ANOVA for 6-PGAD activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 9.838419 | 3 | 3.279473 | 65.6469 | 1.94E-06 | 3.862539 |
| Between tissues | 15.15852 | 3 | 5.05284 | 101.1453 | 2.99E-07 | 3.862539 |
| Error | 0.449606 | 9 | 0.049956 | | | |
| Total | 25.44654 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

Two way ANOVA (Table 2.11 a) revealed an overall significant increase in 6-phosphogluconate dehydrogenase activity between different concentrations of WAF with that of control. Further LSD analysis (Table 2.15 b) revealed that there was a significant increase between 1.5ppm, 3.0ppm and 5.0ppm WAF treated groups with the control and also among

themselves. LSD analysis (Table 2.15 c) also revealed that there was a significant difference between tissues except between muscle and gill.

**Table 2.12 Effect of different concentrations of WAF exposure on the levels of Succinate Dehydrogenase activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 0.603 ± 0.019 | 0.221 ± 0.032 | 0.245 ± 0.045 | 0.403 ± 0.031 |
| 1.5ppm | 0.544 ± 0.029 | 0.132 ± 0.023 | 0.189 ± 0.022 | 0.302 ± 0.010 |
| 3.0ppm | 0.425 ± 0.048 | 0.102 ± 0.011 | 0.154 ± 0.026 | 0.263 ± 0.038 |
| 5.0ppm | 0.384 ± 0.021 | 0.093 ± 0.013 | 0.111 ± 0.011 | 0.223 ± 0.031 |

⇒ Values are expressed as μ moles of formazan formed / mg protein / h.

⇒ Average of six values in each groups ± SD.

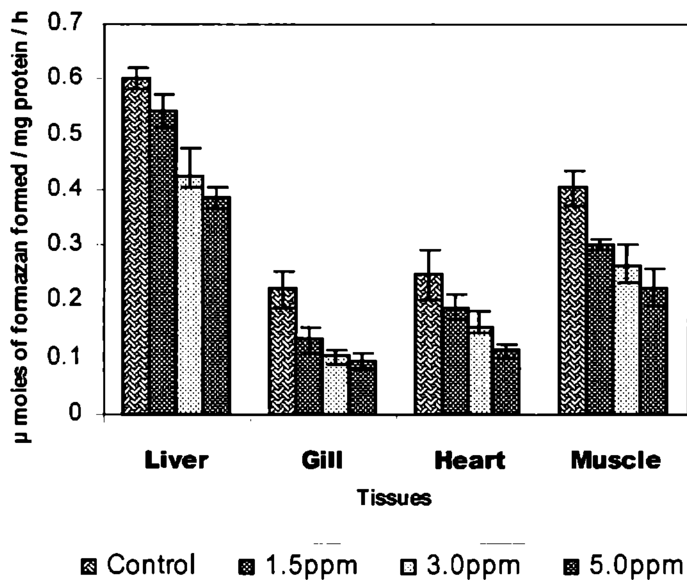



**Figure 2.10 Activity of succinate dehydrogenase in the various tissues exposed to different concentrations of WAF**

**Table 2.12 a Two-Factor ANOVA for Succinate Dehydrogenase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 0.06268 | 3 | 0.020893 | 30.19644 | 4.99E-05 | 3.862539 |
| Between tissues | 0.301628 | 3 | 0.100543 | 145.3105 | 6.11E-08 | 3.862539 |
| Error | 0.006227 | 9 | 0.000692 | | | |
| Total | 0.370536 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

There was an overall significant decrease in the activity of SDH ($P<0.001$) between control and different concentrations of WAF treated groups (Table 2.12 a). Subsequent LSD analysis (Table 2.15 b) revealed that all the concentrations showed significantly decrease in SDH activity when compared to control and also among themselves. However there was no significant difference between 5.0ppm and 3.0ppm WAF treated groups. Further comparison by LSD (Table 2.15 c) between tissues also showed a significant difference ($P<0.001$). Hepatic tissue showed maximum decrease followed by muscle, cardiac and gill tissues. However there was no significant difference between gill and cardiac tissues at 5% level.

**Table 2.13 Effect of different concentrations of WAF exposure on the levels of Malate Dehydrogenase (MDH) activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 0.781 ± 0.025 | 0.237 ± 0.031 | 0.294 ± 0.008 | 0.198 ± 0.008 |
| 1.5ppm | 0.731 ± 0.005 | 0.201 ± 0.030 | 0.265 ± 0.025 | 0.179 ± 0.025 |
| 3.0ppm | 0.684 ± 0.031 | 0.198 ± 0.017 | 0.223 ± 0.017 | 0.131 ± 0.033 |
| 5.0ppm | 0.652 ± 0.015 | 0.155 ± 0.025 | 0.196 ± 0.010 | 0.111 ± 0.008 |

⇒ Values are expressed as μ moles of formazan formed / mg protein / h.

⇒ Average of six values in each groups ± SD.

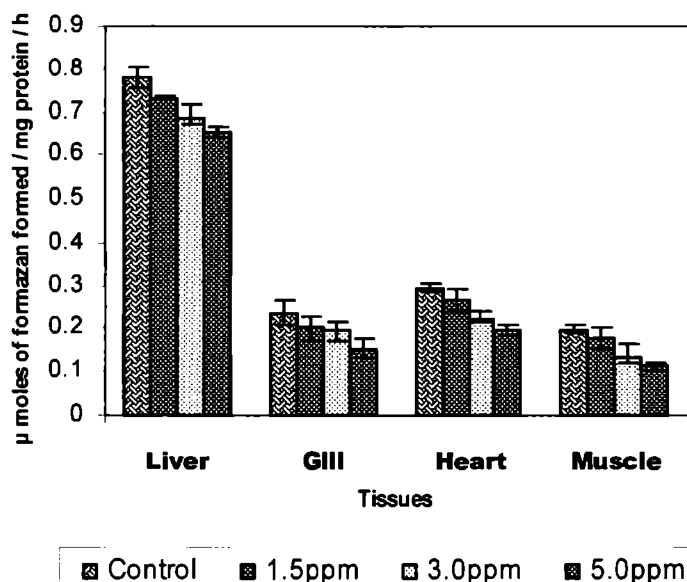



**Figure 2.11 Activity of malate dehydrogenase in the various tissues exposed to different concentrations of WAF**

**Table 2.13 a Two-Factor ANOVA for Malate Dehydrogenase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 0.022061 | 3 | 0.007354 | 45.78554 | 8.94E-06 | 3.862539 |
| Between tissues | 0.805627 | 3 | 0.268542 | 1672.002 | 1.13E-12 | 3.862539 |
| Error | 0.001445 | 9 | 0.000161 | | | |
| Total | 0.829133 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

There was an overall significant decrease in MDH activity (P<0.001) between different concentrations of WAF and control group (Table 2.13 a ) by Two- way ANOVA. Subsequent comparison by LSD analysis (Table 2.13 b) revealed that there was a significant difference (P<0.001) between control and 1.5ppm, 3.0ppm and 5.00ppm WAF treated groups. Comparisons between concentrations also revealed significant difference.

LSD value at 5% level was 0.0202. Comparison between tissues by LSD analysis (Table 2.15 c) reflected significant difference (P<0.001). Hepatic tissue showed maximum decrease and minimum was by muscle tissue.

**Table 2.14 Effect of different concentrations of WAF exposure on Isocitrate dehydrogenase activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 1.02 ± 0.08 | 0.54 ± 0.02 | 0.98 ± 0.09 | 0.57 ± 0.05 |
| 1.5ppm | 0.99 ± 0.07 | 0.51 ± 0.03 | 0.97 ± 0.05 | 0.58 ± 0.06 |
| 3.0ppm | 0.91 ± 0.09 | 0.47 ± 0.05 | 0.94 ± 0.02 | 0.47 ± 0.009 |
| 5.0ppm | 0.80 ± 0.04 | 0.39± 0.01 | 0.85 ± 0.04 | 0.36 ± 0.007 |

⇒ Values are expressed as μg NAD reduced / 30 sec / mg protein.

⇒ Average of six values in each groups ± SD.

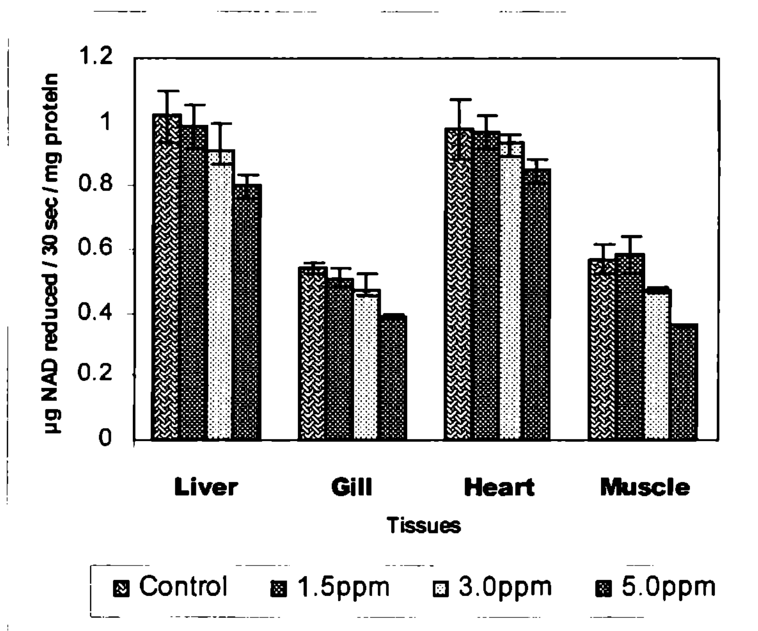



**Figure 2.12 Activity of isocitrate dehydrogenase in the various tissues exposed to different concentrations of WAF**

**Table 2.14 a Two-Factor ANOVA for Isocitrate dehydrogenase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 0.078269 | 3 | 0.02609 | 41.88294 | 1.3E-05 | 3.862539 |
| Between tissues | 0.797219 | 3 | 0.26574 | 426.6054 | 5.13E-10 | 3.862539 |
| Error | 0.005606 | 9 | 0.000623 | | | |
| Total | 0.881094 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 2.14 a) revealed an overall significant decrease (P<0.001) in isocitrate dehydrogenase activity between different WAF concentrations with that of control. Subsequent comparisons by LSD analysis (Table 2.15 b) revealed that there was no significant difference between 1.5ppm WAF treated group with that of control. Whereas all other concentrations significantly differed from one another and also with control. LSD analysis also revealed (Table 2.15 c) a significant difference between tissues except between liver and heart as well as muscle and gill.

**Table 2.15 Effect of different concentrations of WAF exposure on the levels of Cytochrome-c-oxidase activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 100.45 ± 3.23 | 91.02 ± 2.80 | 96.25 ± 2.88 | 74.26 ± 1.33 |
| 1.5ppm | 94.89 ± 4.31 | 82.53 ± 2.07 | 92.58 ± 3.75 | 71.98 ± 2.45 |
| 3.0ppm | 89.65 ± 2.87 | 79.88 ± 2.57 | 89.69 ± 4.04 | 68.58 ± 1.52 |
| 5.0ppm | 84.69 ± 3.71 | 76.77 ± 3.25 | 87.25 ± 1.76 | 65.41 ± 1.80 |

⇒ Values are expressed as μ moles of formazan formed / mg protein / h.

⇒ Average of six values in each groups ± SD.

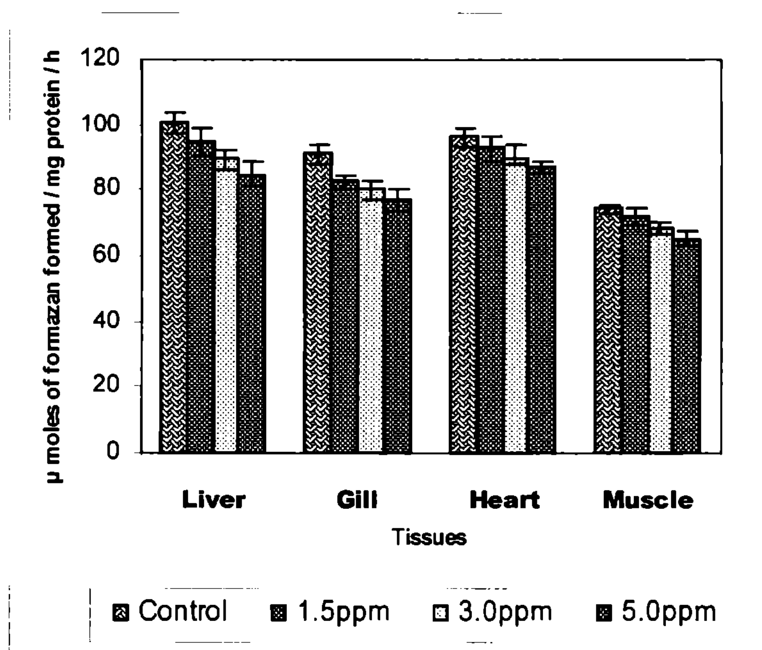



**Figure 2.13 Activity of cytochrome-c-oxidase in the various tissues exposed to different concentrations of WAF**

**Table 2.15 a Two-Factor ANOVA for cytochrome-c-oxidase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 313.8412 | 3 | 104.6137 | 35.4983 | 2.58E-05 | 3.862539 |
| Between tissues | 1290.88 | 3 | 430.2933 | 146.0103 | 5.98E-08 | 3.862539 |
| Error | 26.52306 | 9 | 2.947006 | | | |
| Total | 1631.244 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two way ANOVA (Table 2.13 a) revealed that there was an overall significant decrease (P<0.001) in cytochrome-c-oxidase activity between control and WAF treated groups. Further comparison using LSD analysis revealed (Table 2.15 b) that the concentrations differed significantly (P<0.001) from one another and also with control. LSD value at 5% level was 2.7457. Further comparison by LSD (Table 2.15 c) between tissues revealed

that hepatic and gill tissues showed maximum decrease than cardiac and muscle tissues. However there was no significant difference between hepatic and cardiac tissues.

**Table 2.15 b Results of LSD analysis for different WAF concentrations**

| GROUPS | PARAMETERS | | | | |
|---|---|---|---|---|---|
| | 6PGD | SDH | MDH | ICDH | CCO |
| Control Vs 1.5ppm | P< 0.05 | P < 0.01 | P < 0.01 | NS | P < 0.01 |
| Control Vs 3.0ppm | P < 0.001 | P < 0.05 | P < 0.05 | P < 0.01 | P < 0.05 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.05 | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 1.5ppm | P < 0.05 | P < 0.01 | P < 0.05 | P < 0.05 | NS |
| 5.0ppm Vs 1.5ppm | P < 0.001 | P < 0.01 | P < 0.05 | P < 0.001 | P < 0.01 |
| 5.0ppm Vs 3.0ppm | P < 0.05 | NS | P < 0.01 | P < 0.01 | P < 0.01 |

**Table 2.15 c Results of LSD analysis for different tissues.**

| GROUPS | PARAMETERS | | | | |
|---|---|---|---|---|---|
| | 6PGD | SDH | MDH | ICDH | CCO |
| Liver Vs Gill | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.05 |
| Liver Vs Heart | P < 0.001 | P < 0.001 | P < 0.001 | NS | NS |
| Liver Vs Muscle | P < 0.001 | P < 0.05 | P < 0.001 | P < 0.001 | P < 0.001 |
| Heart Vs Gill | P < 0.05 | NS | P < 0.01 | P < 0.001 | P < 0.05 |
| Muscle Vs Gill | NS | P < 0.01 | P < 0.01 | NS | P < 0.001 |
| Muscle Vs Heart | P < 0.05 | P < 0.01 | P < 0.05 | P < 0.001 | P < 0.01 |

6PGD – 6-phosphogluconate dehydrogenase
SDH – Succinate dehydrogenase
NS – Not significant
ICDH – Isocitrate dehydrogenase
MDH – Malate dehydrogenase
CCO – Cytochrome-c -oxidase

## 2D. DISCUSSION

The foregoing results imply that exposure of *O. mossambicus* to varying concentrations of water-accommodated fractions of crude oil for 21 days has profound influence on the carbohydrate metabolism. The increased surfacing and a decrement in opercular movement as well as copious secretion of mucous of the experimental animals exposed to 3.0ppm and 5.0ppm of water-accommodated fractions of crude oil suggests a severe respiratory stress.

A significant dose-dependent decrement in the total carbohydrates and glycogen content but a steady increase in blood glucose was registered in the experimental fish when exposed to 1.5ppm, 3.0ppm and 5.0ppm concentrations of water-accommodated fractions of crude oil. The tissue wise decreasing trend for total carbohydrates and glycogen is as follows: Liver>Muscle>Heart>Gill. From the above trend, it can be easily visualized that there is a rapid utilization of total carbohydrates and glycogen by all tissues to meet higher energy demands to mitigate the stress caused by water-accommodated fractions of crude oil. Since total carbohydrates represent mostly glycogen it can be presumed that these carbohydrates that are convertable or related to glycogen are effectively tapped under the impact of petroleum hydrocarbons. The present observations also agree with earlier reports on petroleum hydrocarbons toxicity in various aquatic organisms. It has been reported that decrease in glycogen content was observed in crude oil-exposed Atlantic croaker, *Micropogon undulatus L.* (Jo Ann Eurell, 1981). Similar depletion in energy reserves was noticed by

Duncan *et al.* (2002) in *Colossoma macropomum;* Val *et al.* (1999) in some fish species of the Amazon and Sabo *et al.* (1975) in the marine fish *Fundulus heteroclitus*-under the impact of petroleum hydrocarbons. The most profound physiological disturbance in fish exposed to water-accommodated fractions was a dramatic decline in blood oxygen content (Alkindi *et al.*, 1996 and Brauner *et al.,* 1999). Since anoxia or hypoxia are known to elevate carbohydrate and glycogen consumption (Dezwaan and Zandee, 1972) the observed decrease in the levels of the total carbohydrates and glycogen obviously suggests existence of anoxia or hypoxic condition at the tissue level forcing the animal to augment its energy sources.

Interestingly, the decrease in tissue glycogen content of fish exposed to different concentrations of water-accommodated fractions of crude oil shows relationship with an increase in the blood glucose level and the maximum increase in blood glucose was exhibited by 5.0ppm dosed experimental animals, thereby indicating increased breakdown of glycogen to glucose and its mobilization to other tissues to meet energy crisis (Pickering, 1993). Increase in blood glucose level was also reported by several workers under petroleum hydrocarbon toxicity. In support of our data, Prasad *et al.* (1987), reported that exposure of cat fish, *Heteropneustes fossilis* to different concentrations of crude oil extract for a varying period resulted in hyperglycemia. Similar results have been reported by Alkindi *et al.* (1996) that Flounders, *Pleuronectes flesus* were exposed to a 50% dilution of the water -soluble fraction of Omani crude oil also exhibits increased blood sugar level. Omoregia (2002) also reported a similar result in Nile tilapia, *Oreochromis niloticus (L.)* when exposed to

water-soluble fraction of crude oil. Since crude oils are known to effect tissue damage (Khan, 1995 and Marty *et al.*, 1999) as is evident in the necrosis of the gill on histopathological examinations in our study (Chapter 7), the increase in blood glucose may also be due to decrease in glycogen synthesizing potentiality of the tissues as a consequence of cellular damage. Recent studies have demonstrated that cortisol has a direct effect on carbohydrate metabolism, stimulating glycogenolysis and gluconeogenesis (Vijayan *et al.*, 1994, Wright *et al.*, 1989 and Vijayan and Moon, 1994). Gill hyperplasia has been demonstrated in fish exposed to petroleum hydrocarbons (Gardner, 1976) and it is also evident from our histopathological studies (Chapter 7). This suggests that the gills and olfactory cells of the Mullet, *Mugil cephalus* may have been irritated by the noxious volatile components of crude oil causing stimulation of ascending neural pathways and subsequent activation of the hypothalamo-pituitary-interrenal axis. Such a mechanism would account for the rapid rise in the circulating cortisol (Thomas *et al.*, 1980 and Reid *et al.*, 1992). High cortisol concentrations were associated with hyperglycemia in *Fundulus heteroclitus* exposed continuously either to naphthalene or crude oil extracts (DiMichele and Taylor, 1978). Therefore, it can be inferred that increased levels of cortisol is another reason for the hyperglycemia in petroleum hydrocarbon exposed experimental animals.

A significant increase in Lactate dehydrogenase (LDH) and lactic acid content; and a significant decrease in pyruvate were observed with increasing concentrations of water-accommodated fractions of crude oil in liver, gill, heart and muscle tissues of *O.mossambicus*. The orders of

increase in LDH activity in the four different tissues of fish subjected to three different exposures are as follows. Liver > Muscle > Heart > Gill. Nugegoda (2001) have reported a similar increase in LDH activity in juvenile *Australian Bass* and *Macquaria novemaculeata* in response to two different crude oil spills. Similar stimulation of LDH activity in tissues of Nile Tilapia treated with petroleum hydrocarbon effluent (Omoregia, 1997) also favors our findings. Increased LDH activity, besides indicating cell necrosis (evident in chapter 7) as pointed by Nemcso'k *et al.* (1985) may be also due to the injuries of gill epithelium resulting in oxygen depletion in toxicant-exposed experimental animals. The increase in LDH activity also suggests a significant increase in the conversion of pyruvate to lactic acid, thereby leading to the accumulation to lactic acid. In consonance with the increase in LDH activity, a concomitant increase in lactic acid is also observed in all the four tissues under three different exposures. The order of increase in lactic acid content in the four tissues of fish is as follows: Muscle > Liver > Gill > Heart. In agreement with our result, an elevation of lactic acid was reported in *Colossoma macropomum* after exposure to crude oil (Wallice, 1998). The increase in LDH activity with a consequent increase in the levels of lactate suggests the tendency of shift in the metabolism of carbohydrates more towards anaerobic segment, glycolysis than aerobic oxidation, Kreb's cycle (Skidmore, 1970). The other possible reason for the lactate production may be due to its involvement in osmoregulation. During crude oil toxicity loss of monovalent and divalent cations is known to occur. The decrease in osmolarity of the internal media, due to loss of its cations was reported to be compensated by

a concomitant increase in organic ions like lactate, pyruvate, amino acids etc (Alkindi *et al.,* 1996 and Brauner, 1999).

The increase in lactic acid corroborates with the corresponding decrease in pyruvate content of the tissues. The order of decrease in pyruvate is as follows: Liver > Muscle > Heart > Gill. The reason for the observed decrease in pyruvate level indicates its forming a precursor for many metabolic products. The decrease also could be due to its conversion to lactic acid, lipids, triglycerides and glycogen synthesis in addition to its role as a detoxification factor in ammonia toxicity (Sambasiva Rao, 1984).

Phosphorylase 'a' (active form) is significantly elevated with a very significant decrement of phosphorylase 'b' (inactive form) in all the four tissues of fish exposed to 1.5ppm, 3.0ppm and 5.0ppm water-accommodated fractions of crude oil and the maximum increase in phosphorylase 'a' and maximum decrease in phosphorylase 'b' activities were registered in 5.0ppm dosed experimental animals when compared to control, suggesting rapid conversion of inactive phosphorylase 'b' to active phosphorylase 'a' augmented by impact of water-accommodated fractions of crude oil. The tissue specific increase in phosphorylase 'a' activity and decrease in phosphorylase 'b' activity shows the following trend: Liver > Heart > Muscle > Gill. Since petroleum hydrocarbons are known to increase the activities of hormones like adrenaline, noradrenaline and cortisol (Alkindi *et al.,* 1996; Nekvasil and Olson, 1986; Perry and Reid, 1992; Vijayan and Moon, 1994), thereby these hormones activate cyclic AMP production which in turn regulates the inter-conversion of phosphorylase 'b' to phosphorylase

'a' (Robert *et al.*,1998). The higher activity of phosphorylase 'a' over phosphorylase 'b' is agreeable in the tissues of fish exposed to water-accommodated fractions of crude oil which leads to increased conversion of glycogen to glucose-6-phosphate to meet higher energy demands. Elevated levels of phosphorylase 'a' cannot be ascribed only for metabolizing glycogen as it is also necessary for forming pentose sugars through HMP shunt pathway for nucleic acid synthesis. The increase in phosphorylase 'a' activity also suggests increased conversion of inactive phosphorylase 'b' into active phosphorylase 'a' thereby qualitatively elevating phosphorylase 'a' to facilitate the optimal expression of the enzyme activity to mitigate the energy demands. This observation is in agreement with the result of Michaelidis *et al.* (1994) that showed that stress induced anoxia or hypoxia significantly increase the breakdown of glycogen through increased activity of glycogen phosphorylase 'a' in freshwater mussel, *Adonata cygnea (L).*

A significant increase in the activities of glucose-6-phosphate dehydrogenase (G-6PD) and 6-phospho gluconate dehydrogenase (6-PGAD) in all the four tissues of the *O.mossambicus* on exposure to 1.5ppm, 3.0ppm and 5.0ppm of water-accommodated fractions of crude oil and it was observed that the increase was dose-dependent and highest activities were noticed in 5.0ppm exposed animals. The tissue wise increasing trend in G-6PD is as follows: Liver > Heart > Muscle > Gill; whereas for 6-PGAD, the trend is Liver > Heart > Gill > Muscle. Increased activities of these two lipogenic enzymes indicate enhanced oxidation of glucose through HMP pathway to meet energy crisis which provides NADPH for biosynthesis of fatty acids and cholesterol. The pathway supplement 5-carbon sugar residue, ribulose-5-phosphate for

nucleotide and nucleic acid synthesis. These observations support a hypothesis that exposure to petroleum hydrocarbons increased mixed-function oxidase activity (Jo Ann Eurell, 1981). Moore *et al.* (1980) working on *Mytilus edulis* exposed to low concentration of water-accommodated fractions of North Sea crude oil reported similar increase in Glucose-6-phosphate dehydrogenase and 6-phospho gluconate dehydrogenase. This is in agreement with our present observations.

A significant inhibition of succinate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase activities of all the four tissues was observed on exposure to 1.5ppm, 3.0ppm and 5.0ppm of water-accommodated fractions of Bombay High crude oil and the maximum inhibition was exhibited by 5.0ppm dosed hepatic tissue of the experimental animals. The tissue specific lyotropic series in the decrease of succinate dehydrogenase is Liver > Muscle > Heart > Gill; for malate dehydrogenase Liver > Gill > Heart > Muscle and for isocitrate dehydrogenase Liver> Heart > Muscle > Gill. The inhibition of succinate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase activities in the present study reflects decreased turn over of the carbohydrates and thereby energy output, similar to the findings of Gupta *et al.* (1994). Similar inhibition of these three oxidative enzymes was noticed in American oysters and brown shrimp following chronic exposure to crude oil in a simulated ecosystem (Janice *et al.*, 1979). Decreased activities of these oxidative enzymes leads to a concomitant decrease in oxidative phosphorylation and it will decrease the production of NADH and $FADH_2$

through TCA cycle leading towards anaerobiosis. This may be the consequence of superimposed hypoxia by petroleum hydrocarbons toxic environment through inhibition of oxygen uptake capacity of the fish (Alkindi *et al.*,1996). The depletion of oxidative enzymes would impair the energy supply and subsequently lead to cellular degeneration, as is evident from our histopathological studies (Chapter 7). Rudolph *et al.* (2002), Khan (1995) and Marty *et al.* (1999) have reported that the inhibitory activities of these enzymes are due to damage of mitochondrial membrane under the petroleum hydrocarbon stress. The elevation of lactate and lactate/pyruvate ratio with subsequent inhibition of succinate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase activities suggests that the anaerobic metabolism is accelerated due to energy demands as suggested by decreased oxidative enzyme levels (Cederbaum *et al.*, 1974). Since these enzymes are the enzymes of significance in the oxidative metabolism of the cell, any change in their activity is likely to disturb the harmony and co-ordination of the TCA cycle and other metabolic pathways.

Cytochrome-c-oxidase (CCO) activity showed a significant inhibition with increasing concentration of water-accommodated fractions when compared with the control in all the four tissues under different concentrations. In agreement with our results, a significant decrease in CCO activity was reported in gills and liver of fish exposed to water-accommodated fractions of Bass Strait crude oil and chemically dispersed crude oil (Cohen *et al.*, 2001). The decrease in CCO activity suggests mitochondrial electron transfer system to be affected under crude oil impact, leading to reduced synthesis of ATP molecules. Similar trends in cytochrome-c-oxidase activity

have been reported in *Hoplosternum littorale* and *Colossoma macropomum*, when they were exposed to Amazon crude oil (Duncan, 2002). Brown *et al.* (1996) also reported the effect of Exxon Valdez oil spill on the inhibition of cytochrome-c-oxidase activity in *Clupea Pallasi*. The decrease in cytochrome-c-oxidase synchronizes with the decreasing trend in the activities of succinate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase which are mitochondrial enzymes. From the tissue point of view, the effect of water-accommodated fractions of Bombay High crude oil is maximally felt in the liver tissue at 5.0ppm concentration followed by heart, gill and muscle tissues. It was reported by Alkindi *et al.* (1996) Saha *et al.* (1986), Driedzic (1996), Brauner *et al.* (1999) that petroleum hydrocarbons influence a significant inhibition in the whole animal and tissue respiratory rates, it is quite likely that the decrease in cytochrome-c-oxidase might be the result of reduced availability of oxygen which in turn has reduced the capacity of electron transport system to produce ATP molecules (Thomas *et al.*, 1997). In all the four tissues the decrement in cytochrome-c-oxidase activity remained consistently higher in fish exposed to 5.0ppm of water-accommodated fractions of crude oil than in fish exposed to either 3.0ppm or 5.0ppm. The high toxicity of 5.0ppm concentration of water-accommodated fractions might be the consequence of higher respiratory stress (Vanderkooi *et al.*, 1991) by reducing the oxygen utilization capacity both at the whole animal and tissue level.

In this perspective backdrop, it may be concluded that the toxicity of water-accommodated fractions of Bombay High crude oil decreased the ability of animal to respire thereby decreasing oxygen uptake. At the same

time, the requirements of energy are geared up by starting the mobilization of the carbohydrate reserves for production of energy. At this moment, the fish faces a strange situation wherein its energy requirements are elevated at the same time depriving the ability of animal or its tissue for oxygen uptake. Such a situation might lead to a shift in the metabolism tending more towards anaerobic mode of respiration. The suppression of oxidative enzymes may limit the Kreb's cycle thus reducing generation of ATP by the aerobic mode of respiration. Thus the present findings are suggestive of metabolic block somewhere between the glycolytic and TCA cycle pathways inhibiting the flux of glycolytic end products into oxidative cycle, after exposure to petroleum hydrocarbons.

# *Chapter* 3

# Effects Of Petroleum Hydrocarbons On Nitrogen Metabolism

## 3A. INTRODUCTION

Excretion of nitrogen is a liable character and the pattern may change with the life cycle, availability of water, nutrition and water environmental factors (Prosser, 1973). Nitrogenous excretory products are derived from the catabolism of proteins, by way of amino acids which may be transaminated and deaminated or by the degradation of nucleic acids (Bayne *et al.*, 1985). No organism limits its nitrogen excretion to one product, but it is well established that aquatic ones commonly excrete much of their nitrogen mostly in the form of ammonia. It is appeared that the balance between the nitrogen intake and output will reveal significant changes in protein metabolism. Nitrogen balance may be defined as the quantitative difference between nitrogen intake and output. The nitrogen balance plays a significant role in maintaining the dynamic equilibrium. The main end product of nitrogen metabolism in fish is ammonia, which accounts from 55 to 80% of the total nitrogen excreted (Walton and Cowley, 1977), while urea excretion is about 6 to 8%. (Vellas and Serfaty, 1974).

Amino transferases are a group of enzymes that catalyse the process of biological transamination. Aspartate aminotransferases (AST) and Alanine aminotransferases (ALT) are the major ones in this group of enzymes and they function as a link between carbohydrate, fat and protein metabolism (Harper,1998) by catalyzing the interconversion of the strategic compounds like α-ketoglutarate and alanine to pyruvate and glutamic acid; and α-ketoglutarate and aspartate to oxaloacetate and glutamic acid respectively. The keto acids are the sources for Kreb's cycle and

gluconeogenesis. These mechanisms provide additional energy to meet the increased demand under stress (Narvia *et al.,* 1997). Liver is rich in Aspartate aminotransferases (AST) and Alanine aminotransferases (ALT) and changes in plasma levels of these enzymes may be indicative of liver dysfunction (Kapila, 1999).

Glutamate dehydrogenase is a mitochondrial enzyme containing zinc. It is one of the primary enzymes concerned with amino acid metabolism and gluconeogenesis. The reversible conversion of L-glutamic acid to α-ketoglutarate- a member of TCA cycle- is mediated by glutamate dehydrogenase. The enzyme serves as a link between the metabolisms of amino acids and carbohydrates (Robert *et al.,*1998).

Proteases are the enzymes which functions in hydrolyzing proteins to free amino acids and small peptides. The levels of protease activity of the tissue have also served as useful indices to monitor stressful conditions (Millward, 1970).

Since the nitrogen intake through nutrition (proteins) and output through excretion (ammonia and urea) is of prime importance in the maintenance of the dynamic equilibrium, this aspect of study was given due emphasis. The present investigation was oriented to determine the levels of proteins, free amino acids, ammonia, urea, and the enzymes like protease, glutamate dehydrogenase and amino transferases in liver, gill, heart and muscle tissues of *O.mossambicus* under the impact to different concentrations of water-accommodated fractions of Bombay High crude oil.

## 3B. MATERIALS AND METHODS

Collection, acclimation of fish, WAF preparation and method of WAF dosing were the same as that described in the Chapter 2B.

### 3B.1 METHODS USED FOR THE BIOCHEMICAL ANALYSIS

The following are the parameters studied under Nitrogen metabolism.

#### a. Estimation of Protein

Protein was estimated by the method of Lowry *et al.* (1951).

#### Reagents

0. 1N NaOH, Alkaline copper reagent, Folin-Ciocalteau phenol reagent, standard protein solution, 10% Tri chloro acetic acid (TCA).

#### Procedure

After isolating the tissues, 2% homogenates were prepared in 0.33 M cold sucrose solution. The homogenates were centrifuged at 1000 g for 15 minutes. To the supernatant equal volume of 10% TCA was added to precipitate the proteins. The contents were allowed to stand for 30 minutes at room temperature and centrifuged at 1000 g for 15 minutes. The sediment was dissolved in 1 ml of 0.1N NaOH. After suitable dilution a known volume of the solution was mixed with 5.0 ml of alkaline copper reagent. Shaken well. After 10 minutes, 0.5 ml of Folin-Ciocalteau Phenol reagent was added and mixed well. The tubes were kept for another 30 minutes.The absorbance was measured at 500 nm, in a spectrophotometer against a reagent blank. The

system devoid of sample was used as the blank. Bovine serum albumin was used as standard. The values were expressed as mg / g wet wt. of tissue.

**b. Estimation of Protease**

Protease activity was estimated by the method of Moore and Stein, 1954.

**Reagents**

100 μ moles of phosphate buffer (pH 7.0), haemoglobin, 10% Tri chloro acetic acid (TCA), Ninhydrin reagent.

**Procedure**

5% homogenate of liver and 10% homogenate of muscle, heart and gill tissues were prepared in cold distilled water. The homogenates were centrifuged at 1000 g for 15 minutes and the supernatants were taken for the assay. The assay mixture of 2.0 ml contained: 100 μ moles of phosphate buffer (pH 7.0), 20 mg of heat-denatured haemoglobin (substrate) and 0.5 ml of homogenate supernatant. The contents were incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 2.0 ml of 10% TCA. The unincubated samples were treated with 2.0 ml of 10% TCA prior to the addition of the supernatant. The contents of both the incubated and unincubated samples were filtered and the amino acid level was determined in the filtrates. To 0.5 ml of the filtrate, 2.0 ml of ninhydrin reagent was added, heated to boiling in a water bath for 5 minutes and cooled. The volume was made up to 10.0 ml with distilled waster and the colour was

measured at 570 nm in spectrophotometer against a reagent blank. All samples were corrected for zero time controls. The blank consists of 0.5 ml distilled water and 2.0 ml of ninhydrin reagent which received the same treatment as that of the samples. The enzyme activity was expressed in µ moles of tyrosine equivalents / mg protein / h.

**c. Estimation of Free Amino Acids (Ninhydrin Positive Substances)**

Total free amino acids also known as Ninhydrin positive substances has been estimated by the method of Moore and Stein, 1954.

**Reagents**

10% Tri chloro acetic acid (TCA), Ninhydrin reagent, standard Tyrosine.

**Procedure**

2% homogenate of liver and 5% homogenates of muscle, heart and gill tissues were prepared in 10% TCA and centrifuged at 1000 g for 15 minutes. To 0.5 ml of the supernatant 2.0 ml of ninhydrin reagent was added. The contents were kept in a boiling water bath for 5 minutes and cooled immediately. After 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 ninydrin reagent, which received the same treatment as that of samples. The amino acid content was expressed as µ moles of tyrosine equivalent / g wet wt. of tissue.

### d. Estimation of Ammonia

The ammonia content was estimated by the method of Bergmeyer, 1965.

#### Reagents

15% perchloric acid (PCA), 15% NaOH, Nessler's reagent.

#### Procedure

10% homogenates of muscle, heart and gill and 5% homogenate of liver tissues were prepared in cold distilled water. The homogenates were centrifuged at 1000 g for 15 minutes. To 0.5 ml of the supernatant, 2.0 ml of 15% perchloric acid (PCA) was added, mixed well and centrifuged at 1000 g for 15 minutes. The residues were discarded and the supernatants were neutralized with 2.0 ml of 15% NaOH. Then 0.5 ml of Nessler's reagent was added and absorbance was read immediately at 495 nm in a spectrophotometer against a reagent blank. The blank was prepared by adding 0.5 ml of Nessler's reagent to 2.0 ml of distilled water. The values were expressed as μ moles of ammonia / g wet wt. of tissue.

### e. Estimation of Tissue and Blood Urea

The urea content was estimated by the method of Natelson, 1971.

#### Reagents

15% perchloric acid (PCA), acid mixture (3 parts of phosphoric acid and 1 part of analar sulphuric acid), 2% diacetyl monoxime, 10% sodium tungstate.

**Procedure**

5% homogenate of liver tissue and 10% homogenates of muscle, heart and gill tissues were prepared in 15% PCA and centrifuged for 15 minutes. Washed 0.1 ml of blood into 3.3 ml of water and added 0.8 ml of 10% sodium tungstate and 0.3 ml of 2/3 N $H_2SO_4$. Mixed well and centrifuged at 1000 g for 15 minutes. To 0.5 ml of the supernatant, 1.0 ml of the acid mixture was added and mixed well. Then 0.5 ml of 2% diacetyl monoxime was added and kept in a boiling water bath for 15 minutes. After cooling the tubes the absorbance was read at 480 nm in a spectrophotometer against a reagent blank. The blank consisted of 0.5 ml of PCA, 1.0 ml of acid mixture and 0.5 ml 2% diacetyl monoxime which received the same treatment as that of the samples. The values were expressed as μ moles of urea / g wet wt. of tissue.

**f. Estimation of Glutamate Dehydrogenase (GDH) (L-glutamate:NAD(P) Oxidoreductase; E.C.1.4.1.3)**

Glutamate dehydrogenase activity was estimated by the method of Lee and Lardy, 1965.

**Reagents**

0.33M sucrose solution, 40 μ moles of sodium glutamate, 100 μ moles of potassium phosphate buffer (pH 7.4), 0.1 μ moles of NAD, 4 μ moles of INT (2-p-idodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride).

**Procedure**

10% homogenate of gill and 5% homogenate of muscle, heart and liver tissues were prepared in cold 0.33 M sucrose solution and centrifuged at

1000 g for 15 minutes. The supernatants were used for assay. The reaction mixture of 2.0 ml contained; 40 μ moles of sodium glutamate, 100 μ moles of potassium phosphate buffer (pH 7.4), 0.1 μ moles of NAD, 4 μ moles of INT and 0.5 ml of homogenate supernatant. The subsequent steps followed were the same as described for the SDH. The enzyme activity was expressed in μ moles of formazan formed / mg protein / h.

**g. Estimation of Aspartate aminotransferase (AST) ( L- aspartate: 2-oxoglutarate aminotransferase; E.C. 2.6.1.1) in serum and other tissues**

L-Aspartate aminotransferase (AST) was assayed by the method of Mohun and Cook, 1957

**Reagents**

Buffered substrate (0.1 M phosphate buffer (pH 7.4), 1M aspartic acid, 2 mM 2-oxoglutarate, 2, 4-Dinitro phenyl hydrazine (DNPH), 0.4 N NaOH, pyruvate standard, 0.33 M sucrose.

**Procedure**

10% homogenate of muscle, heart and gill and 5% homogenate of liver tissues were prepared in 0.33 M cold sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatant obtained was used as the enzyme source. Pipetted out 1 ml each of buffered substrate into two test tubes labelled 'test' and 'control' Added 0.2 ml of the enzyme / serum to the tube labelled 'test' and incubated the tubes at 37°C for 60 minutes. After incubation, 0.2 ml of the enzyme / serum was added to the control tube. 1 ml

of 2, 4 -DNPH reagent was added to each tube and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 520 nm in a spectrophotometer against a blank. The blank preparation was the same as that of the experimental, except the corresponding volume of distilled water substitutes the supernatant. The AST activity for tissue and serum were expressed as units / min /mg protein and units / L respectively.

**h. Estimation of Alanine aminotrasaminase (ALT) ( DL- alanine: 2-oxoglutarate aminotransferase; E.C. 2.6.1.2) in serum and other tissues**

Alanine aminotransferase was estimated by the method of Mohun and Cook, 1957

**Reagents**

Buffered substrate (0.1 M phosphate buffer, pH 7.4; 0.2 M DL-alanine, 2mM 2-oxoglutarate), 2, 4 -Dinitro phenyl hydrazine (DNPH), 0.4 N NaOH, standard pyruvate solution.

**Procedure**

10% homogenate of liver, muscle, gill and heart tissues were prepared in 0.33 M sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatants were used for assay. Pipetted out 1 ml each of buffered substrate into two test tubes labelled 'test' and 'control' Added 0.2

ml of the supernatant / serum to the tube labelled 'test' and incubated the tubes at 37°C for 30 minutes. After incubation, 0.2 ml of the enzyme / serum was added to the control tube. 1 ml of 2, 4 - DNPH reagent was added to each tube and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 520 nm in a spectrophotometer. The values of tissue and serum ALT were expressed as units / min / mg protein and units / L respectively.

## 3C. RESULTS

Effect of exposure to different concentrations of WAF for three weeks on the protein content, protease activity, free amino acids, ammonia, tissue urea, blood urea, glutamate dehydrogenase, aspartate transaminase, alanine transaminase in the liver, gill, heart and muscle tissues and serum AST and ALT of *O.mossambicus* are given in Tables 3.1 to 3.10 and in Figures 3.1 to 3.8.

The statistical significance of the results were tested by ANOVA (analysis of variance) followed by LSD (Least Significance Difference) analysis.

**Table 3.1 Effect of different concentrations of WAF exposure on the protein content in different tissues of *O. mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 70.13 ± 5.12 | 54.21 ± 5.62 | 55.77 ± 7.22 | 41.79 ± 4.78 |
| 1.5ppm | 73.24 ± 4.35 | 52.11 ± 6.11 | 50.34 ± 5.93 | 45.24 ± 7 18 |
| 3.0ppm | 54.12 ± 4.83 | 40.25 ± 4.96 | 43.27 ± 6.59 | 33.51 ± 6.23 |
| 5.0ppm | 50.96 ± 5.69 | 37.48 ± 7.25 | 44.54 ± 6.03 | 35.62 ± 5.96 |

⇒ Values are expressed in mg protein / g wet wt. of tissue.

⇒ Average of six values in each groups ± SD.

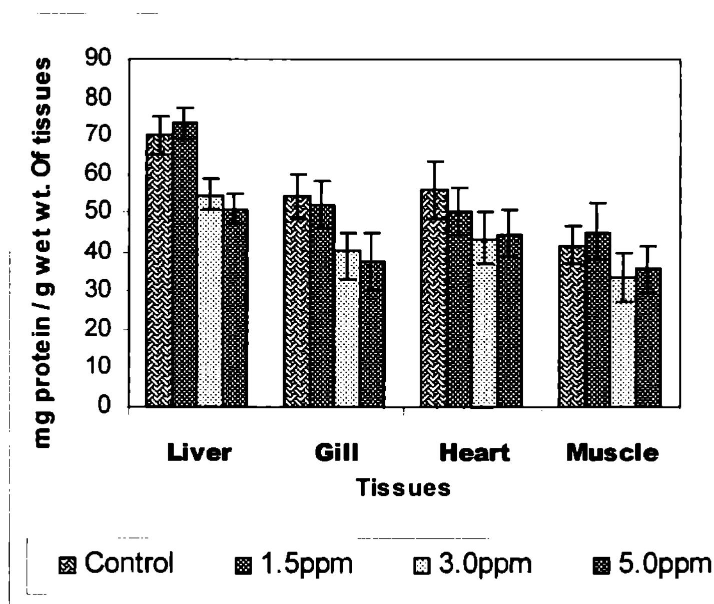



**Figure 3.1 Levels of Total protein in the various tissues exposed to different concentrations of WAF**

**Table 3.1 a Two-Factor ANOVA for Protein**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 665.0233 | 3 | 221.6744 | 18.25713 | 0.000363 | 3.862539 |
| Between tissues | 1121.213 | 3 | 373.7377 | 30.78107 | 4.62E-05 | 3.862539 |
| Error | 109.2762 | 9 | 12.1418 | | | |
| Total | 1895.513 | 15 | | | | |

SS-sum of squares, df-degrees of freedom, MS-mean of squares.

Two-Factor ANOVA (Table 3.1 a) revealed a significant decrease (P<0.001) in protein content between control and all different concentrations of WAF treated groups. Subsequent comparison by LSD analysis (Table 3.4 b) revealed that there was no significant change between control and 1.5ppm WAF treated groups. Whereas comparison between 3.0ppm and 5.0ppm WAF treated groups with the control showed a significant decrease

(P<0.001) in protein content. Comparison between the concentrations showed a significant difference, except between 3.0ppm and 5.0ppm treated groups. LSD analysis (Table 3.4 c) also revealed that there was a significant difference between tissues except between gill and heart.

**Table 3.2 Effect of different concentrations of WAF exposure on the Protease activity in different tissues of *O. mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 1.85 ± 0.13 | 0.94 ± 0.11 | 1.43 ± 0.09 | 1.21 ± 0.08 |
| 1.5ppm | 2.35 ± 0.38 | 1.23 ± 0.18 | 1.84 ± 0.06 | 1.45 ± 0.06 |
| 3.0ppm | 2.81 ± 0.22 | 1.83 ± 0.39 | 2.43 ± 0.28 | 2.01 ± 0.08 |
| 5.0ppm | 3.31 ± 0.44 | 1.91 ± 0.05 | 2.71 ± 0.04 | 2.53 ± 0.41 |

⇒ Values are expressed in μ moles of tyrosine equivalent / mg protein / h.
⇒ Average of six values in each groups ± SD.

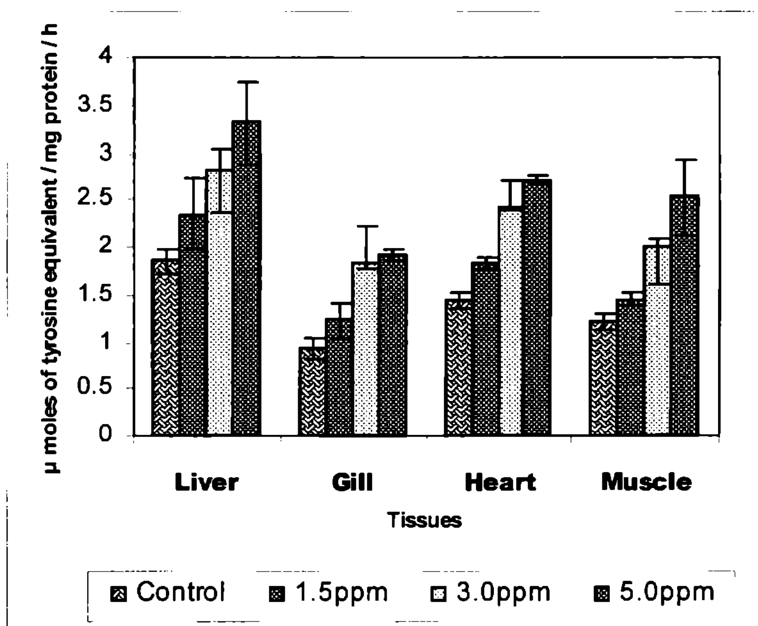



**Figure 3.2 Activity of protease in the various tissues exposed to different concentrations of WAF**

**Table 3.2 a Two-Factor ANOVA for Protease activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 3.77335 | 3 | 1.257783 | 108.0157 | 2.24E-07 | 3.862539 |
| Between tissues | 2.63805 | 3 | 0.87935 | 75.5167 | 1.06E-06 | 3.862539 |
| Error | 0.1048 | 9 | 0.011644 | | | |
| Total | 6.5162 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Two-Factor ANOVA (Table 3.2 a) revealed an overall significant increase ($P<0.001$) in Protease activity in WAF treated groups when compared to control. Further comparison by LSD analysis (Table 3.4 b) revealed that there was a significant increase in protease activity between control and all concentrations of WAF treated groups. Similarly LSD analysis (Table 3.4 c) also revealed that there was a significant difference between tissues also. Liver exhibits greater protease activity, followed by cardiac, muscle and gill tissues.

**Table 3.3 Effect of different concentrations of WAF exposure on the Free Amino acids in different tissues of *O. mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 55.45 ± 5.28 | 9.51 ± 1.85 | 10.69 ± 1.73 | 8.76 ± 1.96 |
| 1.5ppm | 52.34 ± 4.94 | 10.25 ± 2.00 | 9.28 ± 1.05 | 13.27 ± 2.10 |
| 3.0ppm | 57.65 ± 6.07 | 17.35 ± 2.04 | 20.21 ± 3.46 | 19.35 ± 3.04 |
| 5.0ppm | 70.01 ± 8.45 | 19.37 ± 3.47 | 24.59 ± 4.15 | 20.44 ± 3.18 |

⇒ Values are expressed in μ moles of tyrosine / g wet wt. of tissues.

⇒ Average of six values in each groups ± SD.

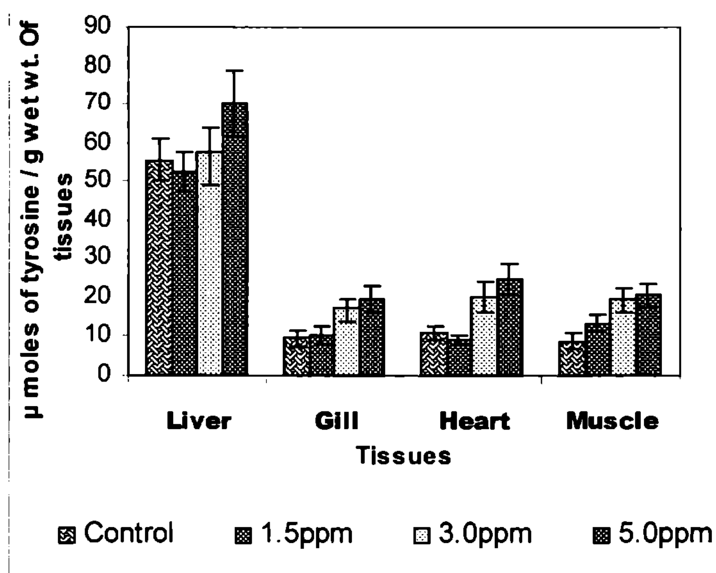



**Figure 3.3 Total free amino acid content in the various tissues exposed to different concentrations of WAF**

**Table 3.3 a Two-Factor ANOVA for Free amino acid**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between | 443.5405 | 3 | 147.8468 | 20.48812 | 0.000233 | 3.862539 |
| concentrations | 5713.453 | 3 | 1904.484 | 263.9171 | 4.36E-09 | 3.862539 |
| Between tissues | 64.946 | 9 | 7.216222 | | | |
| Error | 6221.939 | 15 | | | | |
| Total | | | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Two- Factor ANOVA (Table 3.3 a ) revealed a significant change in the levels of free amino acids between WAF dosed experimental animals and control. Further comparison by LSD analysis (Table 3.4 b) revealed that there was no significant change between control and 1.5ppm treated group, whereas 3.0ppm and 5.0ppm groups showed a significant difference when compared to control. Comparison between tissues by LSD (Table 3.4 c) showed a significant difference only between gill and liver, heart and liver as

well as muscle and liver. Whereas other comparisons did not reveal any significant difference.

**Table 3.4 Effect of different concentrations of WAF exposure on the level of ammonia in different tissues of *O. mossambicus.***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 38.51 ± 1.95 | 22.45 ± 1.25 | 24.06 ± 0.93 | 20.22 ± 0.64 |
| 1.5ppm | 49.23± 3.53 | 29.54 ± 2.12 | 31.25 ± 0.59 | 25.21 ± 0.83 |
| 3.0ppm | 60.03 ± 3.89 | 33.47 ± 0.74 | 35.29 ± 0.88 | 30.22 ± 1.04 |
| 5.0ppm | 65.11 ± 0.91 | 37.25 ± 1.87 | 39.45 ± 1.08 | 35.28 ± 2.07 |

⇒ Values are expressed in μ moles of urea / g wet wt. of tissues.
⇒ Average of six values in each groups ± SD.

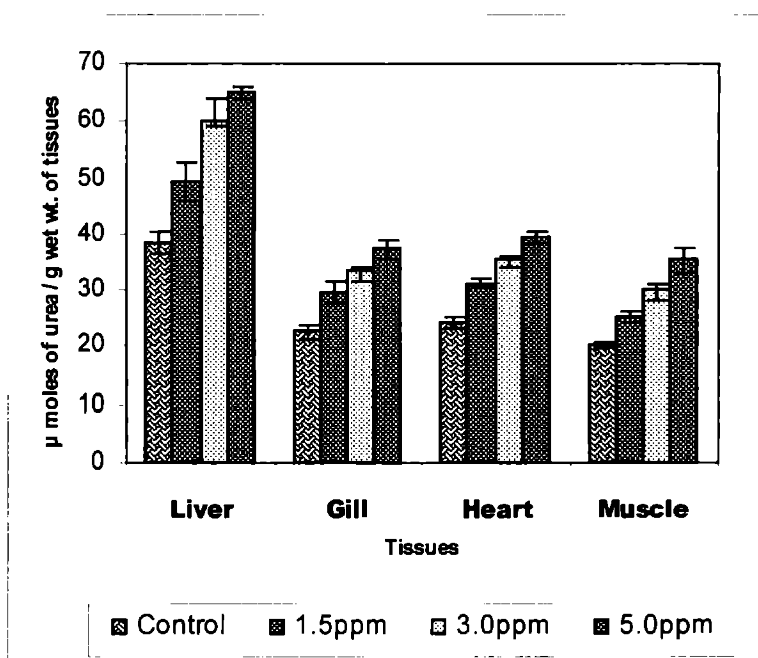



**Figure 3.4 Ammonia content in the various tissues exposed to different concentrations of WAF**

**Table 3.4 a Two-Factor ANOVA for Ammonia content**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 740.3379 | 3 | 246.7793 | 29.97755 | 5.14E-05 | 3.862539 |
| Between tissues | 1608.053 | 3 | 536.0178 | 65.11284 | 2.01E-06 | 3.862539 |
| Error | 74.08923 | 9 | 8.232136 | | | |
| Total | 2422.48 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Two-Factor ANOVA (Table 3.4 a) revealed an overall significant increase (P<0.01) in ammonia formation in all the WAF treated groups when compared to control. Subsequent comparison by LSD analysis (Table 3.4 b) showed that there was a significant increase between control and all WAF dosed animals. Comparison between the concentrations showed that there was a significant increase except between 5.0ppm and 3.0ppm treated groups. LSD analysis also revealed that there was a significant difference between tissues (Table 3.4 c) except between heart and gill as well as muscle and gill.

**Table 3.4 b Results of LSD analysis for different WAF concentrations**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Protein | Protease | Free amino acid | Ammonia |
| Control Vs 1.5ppm | NS | P < 0.05 | NS | P < 0.05 |
| Control Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.01 | P < 0.01 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 1.5ppm | P < 0.01 | P < 0.05 | P < 0.01 | P < 0.05 |
| 5.0ppm Vs 1.5ppm | P < 0.001 | P < 0.05 | P < 0.01 | P < 0.001 |
| 5.0ppm Vs 3.0ppm | NS | P < 0.05 | P < 0.05 | NS |

NS-Not significant

**Table 3.4 c Results of LSD analysis for different tissues.**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Protein | Protease | Free amino acid | Ammonia |
| Liver Vs Gill | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| Liver Vs Heart | P < 0.001 | P < 0.05 | P < 0.001 | P < 0.001 |
| Liver Vs Muscle | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| Heart Vs Gill | NS | P < 0.001 | NS | NS |
| Muscle Vs Gill | P < 0.01 | P < 0.05 | NS | NS |
| Muscle Vs Heart | P < 0.01 | P<0.05 | NS | P < 0.05 |

NS-Not significant

**Table 3.5 Effect of different concentrations of WAF exposure on the urea level in different tissues of *O. mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 13.85 ± 1.75 | 5.34 ± 0.99 | 5.84 ± 0.94 | 4.61 ± 0.81 |
| 1.5ppm | 14.51 ± 2.41 | 4.57 ± 1.02 | 6.91 ± 1.10 | 4.22 ± 0.77 |
| 3.0ppm | 20.34 ± 2.67 | 10.37 ± 1.24 | 12.34 ± 1.12 | 7.35 ± 1.02 |
| 5.0ppm | 22.36 ± 3.33 | 10.99 ± 1.42 | 14.18 ± 2.04 | 8.01 ± 0.97 |

⇒ Values are expressed in µ moles of urea / g wet wt. of tissues.
⇒ Average of six values in each groups ± SD.

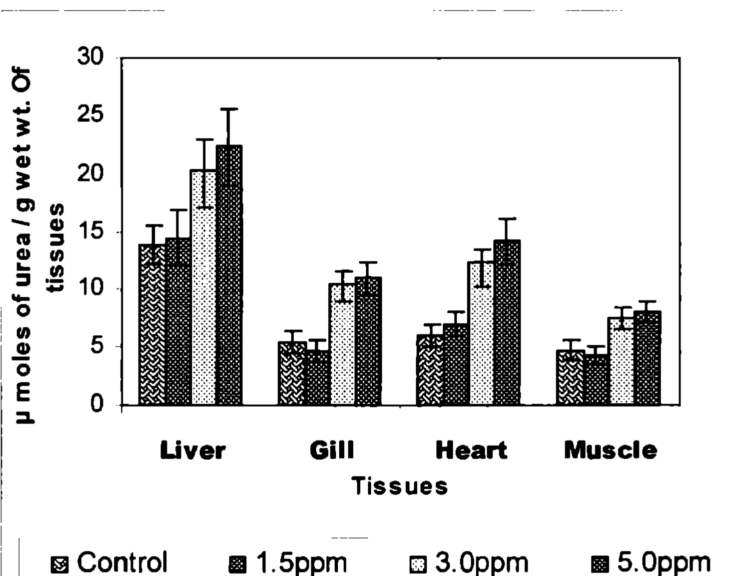



**Figure 3.5 Urea level in the various tissues exposed to different concentrations of WAF**

**Table 3.5 a Two-Factor ANOVA for Urea**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 136.1111 | 3 | 45.37036 | 35.68518 | 2.54E-05 | 3.862539 |
| Between tissues | 320.7611 | 3 | 106.9204 | 84.09615 | 6.66E-07 | 3.862539 |
| Error | 11.24266 | 9 | 1.271406 | | | |
| Total | 468.3148 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Two-Factor ANOVA (Table 3.5 a) revealed a significant difference (P<0.001) in urea content between control and WAF treated groups. Further comparisons by LSD analysis (Table 3.9 b) revealed that comparison between 1.5ppm WAF treated group with that of control did not reveal any significant change. Whereas 3.0ppm and 5.0ppm WAF dosed experimental animals showed a significant difference when compared to control. Comparison between the concentrations revealed a significant change except between 5.0ppm and 3.0ppm treated groups. Comparisons by LSD analysis also revealed (Table 3.9 c) a significant change between tissues except between gill and muscle.

**Table 3.6 Effect of different concentrations of WAF exposure on the activity of Blood urea of *O. mossambicus***

| Control | Concentrations of WAF exposure | | |
|---|---|---|---|
| | 1.5ppm | 3.0ppm | 5.0ppm |
| 11.76 ± 0.74 | 13.68 ± 0.43 | 16.79 ± 0.88 | 20.43 ± 0.71 |

⇒ Values are expressed in mg / 100 ml.

⇒ Average of six values in each groups ± SD.

**Table 3.6 a One way ANOVA for Blood Urea**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 258.9606 | 3 | 86.3202 | 142.6969 | 1.14E-13 | 3.098393 |
| Within concentrations | 12.0984 | 20 | 0.60492 | | | |
| Total | 271.059 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

One way ANOVA revealed a significant increase in blood urea level (F=142.6969, P<0.001) between the experimental groups of animals and control (Table 3.6 a). The highest level was noted in 5.0ppm treated group (173.72% of control) followed by 3.0ppm group (142.77% of the control) and 1.5ppm group (116.33% of control). Subsequent LSD analysis (Table 3.10 c) revealed that the different concentrations of WAF treated groups differed significantly from one another at 5% level.

**Table 3.7 Effect of different concentrations of WAF exposure on the activity of Glutamate Dehydrogenase (GDH) in different tissues of *O. mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 0.0987 ± 0.016 | 0.053 ± 0.009 | 0.0981 ± 0.018 | 0.048 ± 0.008 |
| 1.5ppm | 0.0991 ± 0.012 | 0.055 ± 0.010 | 0.0952 ± 0.014 | 0.051 ± 0.009 |
| 3.0ppm | 0.0812 ± 0.010 | 0.034 ± 0.006 | 0.0782 ± 0.012 | 0.037 ± 0.006 |
| 5.0ppm | 0.0621 ± 0.011 | 0.037 ± 0.006 | 0.0743 ± 0.015 | 0.033 ± 0.005 |

⇒ Values are expressed in µ moles of formazan formed / mg protein / h.

⇒ Average of six values in each groups ± SD.

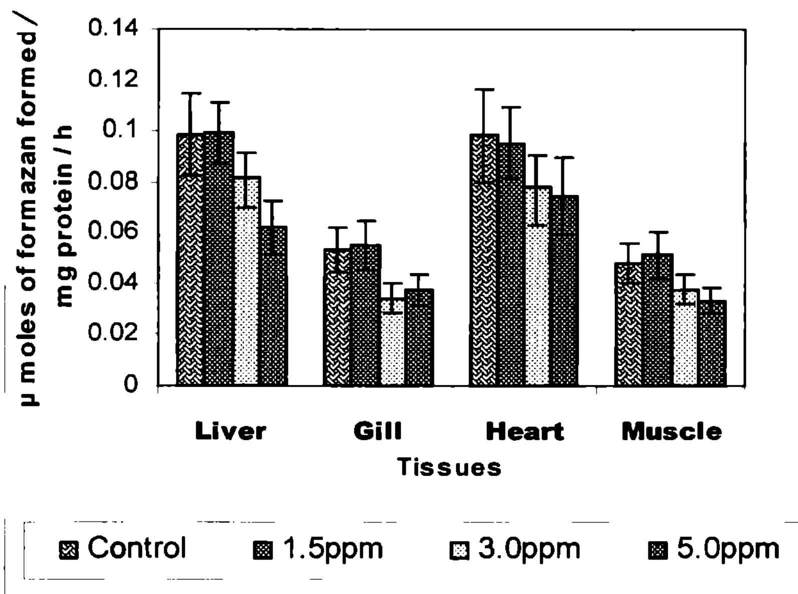



**Figure 3.6 Activity of GDH in the various tissues exposed to different concentrations of WAF**

**Table 3.7 a Two-Factor ANOVA for Glutamate Dehydrogenase**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 0.001699 | 3 | 0.000566 | 22.58851 | 0.000159 | 3.862539 |
| Between tissues | 0.007194 | 3 | 0.002398 | 95.64642 | 3.81E-07 | 3.862539 |
| Error | 0.000226 | 9 | 2.51E-05 | | | |
| Total | 0.009118 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares

Two-Factor ANOVA (Table 3.7 a) revealed that there was a significant inhibition ($P<0.05$) of glutamate dehydrogenase activity in all the WAF treated experimental animals when compared to control group. Subsequent analysis by LSD (Table 3.9 b) revealed that there was no significant difference between control and 1.5ppm WAF treated groups, whereas there was a significant inhibition ($P<0.001$) between 3.0ppm and

5.0ppm WAF treated groups with the control. Comparisons between concentrations showed a significant change, except between 5.0ppm and 3.0ppm WAF treated groups. LSD analysis also (Table 3.9 c) showed that there was a significant difference between tissues except between liver and heart as well as muscle and gill.

**Table 3.8 Effect of different concentrations of WAF exposure on the activity of Aspartate transaminase (AST) in different tissues of *O. mossambicus.***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 1.66 ± 0.19 | 0.94 ± 0.07 | 1.01 ± 0.06 | 1.27 ± 0.31 |
| 1.5ppm | 1.98 ± 0.17 | 1.07 ± 0.11 | 1.17 ± 0.12 | 1.68 ± 0.21 |
| 3.0ppm | 2.44 ± 0.25 | 1.32 ± 0.45 | 1.35 ± 0.09 | 1.99 ± 0.23 |
| 5.0ppm | 3.25 ± 0.29 | 1.89 ± 0.22 | 1.91 ± 0.11 | 2.38 ± 0.42 |

⇒ Values are expressed in units / min / mg protein.

⇒ Average of six values in each groups ± SD.

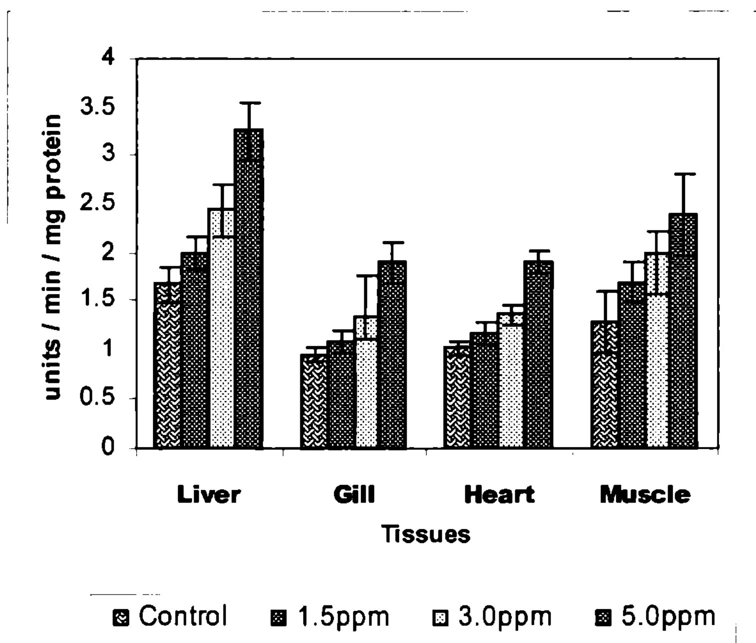



**Figure 3.7 AST activity in the various tissues exposed to different concentrations of WAF**

**Table 3.8a Two-Factor ANOVA for AST activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 2.953819 | 3 | 0.0984606 | 44.07451 | 1.05E-05 | 3.862539 |
| Between tissues | 2.712619 | 3 | 0.904206 | 40.47552 | 1.5E-05 | 3.862539 |
| Error | 0.201056 | 9 | 0.02234 | | | |
| Total | 5.867494 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Tissue Aspartate transaminase activity showed a significant increase (P<0.001) between WAF treated groups and control group as observed by ANOVA (Table 3.8 a). Further comparisons by LSD (Table 3.9 b) analysis revealed that there was a significant difference between control and 1.5ppm, control and 3.0ppm and control and 5.0ppm treated group. LSD value at 5% level was 0.239. Comparisons between tissues by LSD (Table 3.9 c) revealed that liver gave significantly higher values compared with other tissues. Significantly lower value (P<0.001) was observed in gill. But between gill and cardiac tissues there was no significant difference at 5% level.

**Table 3.9 Effect of different concentrations of WAF exposure on the activity of Alanine transaminase (ALT) in different tissues of *O. mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 2.25 ± 0.05 | 0.98 ± 0.01 | 1.68 ± 0.02 | 2.11 ± 0.04 |
| 1.5ppm | 5.12 ± 0.56 | 1.19 ± 0.02 | 2.14 ± 0.17 | 3.24 ± 0.32 |
| 3.0ppm | 6.24 ± 0.82 | 2.55 ± 0.04 | 3.63 ± 0.14 | 4.98 ± 0.11 |
| 5.0ppm | 8.23 ± 0.18 | 3.43 ± 0.03 | 4.38 ± 0.11 | 5.35 ± 0.05 |

⇒ Values are expressed in units / min / mg protein.

⇒ Average of six values in each groups ±SD.

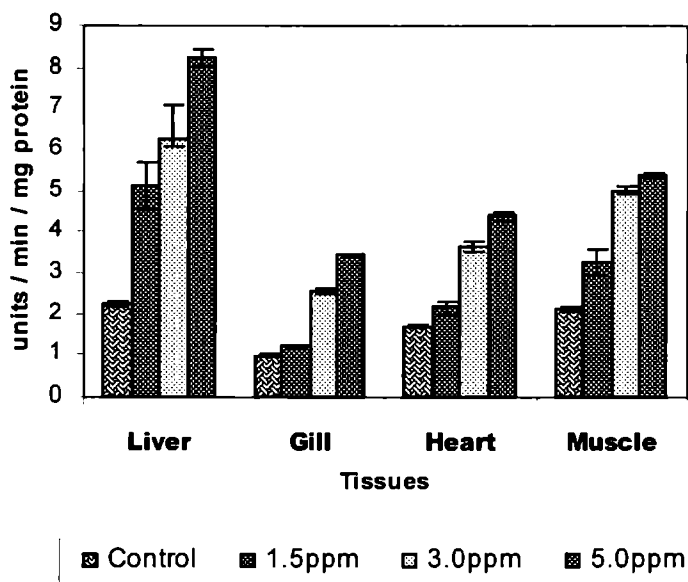



**Figure 3.8 ALT activity in the various tissues exposed different concentrations of WAF**

**Table 3.9 a Two-Factor ANOVA for ALT activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 29.89079 | 3 | 9.963597 | 19.85312 | 0.000263 | 3.862539 |
| Between tissues | 25.64245 | 3 | 8.547482 | 17.03142 | 0.000472 | 3.862539 |
| Error | 4.51679 | 9 | 0.501866 | | | |
| Total | 60.05003 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

There was a significant difference in the activity of Alanine transaminase ($P<0.01$) between the different WAF treated groups and control group as obtained by ANOVA (Table 3.9 a). Further comparisons using LSD analysis (Table 3.9 b) revealed that all the three concentrations selected for the study differed significantly from one another and also with the control. 5.0ppm concentration recorded significantly higher values than other concentrations. LSD value at 5% level was 0.5676. Comparisons between

tissues by LSD analysis (Table 3.9 c) also showed a significant difference (P<0.05). Liver recorded significantly higher values followed by muscle, heart and gill tissues.

**Table 3.9 b Results of LSD analysis for different WAF concentrations**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Urea | GDH | AST | ALT |
| Control Vs 1.5ppm | NS | NS | P < 0.05 | P < 0.01 |
| Control Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.01 | P < 0.001 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 1.5ppm | P < 0.001 | P < 0.001 | P < 0.01 | P < 0.05 |
| 5.0ppm Vs 1.5ppm | P < 0.001 | P < 0.001 | P < 0.01 | P < 0.001 |
| 5.0ppm Vs 3.0ppm | NS | NS | P < 0.01 | P < 0.05 |

NS-Not significant

**Table 3.9 c Results of LSD analysis for different tissues**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Urea | GDH | AST | ALT |
| Liver Vs Gill | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| Liver Vs Heart | P < 0.001 | NS | P < 0.001 | P < 0.001 |
| Liver Vs Muscle | P < 0.001 | P < 0.001 | P < 0.01 | P < 0.01 |
| Heart Vs Gill | P < 0.001 | P < 0.001 | NS | P < 0.05 |
| Muscle Vs Gill | NS | NS | P < 0.01 | P < 0.001 |
| Muscle Vs Heart | P < 0.001 | P < 0.001 | P < 0.05 | P < 0.05 |

NS – Not significant
GDH – Glutamate dehydrogenase
AST – Aspartate transaminase
ALT – Alanine transaminase

**Table 3.10 Effect of different concentrations of WAF exposure on the activity of Serum Aspartate amino transaminase (AST) and Alanine amino transaminase (ALT) in O. *mossambicus.***

| Groups | Control | Concentrations of WAF exposure | | |
|---|---|---|---|---|
| | | 1.5ppm | 3.0ppm | 5.0ppm |
| AST | 49.85 ± 1.56 | 231.69 ± 2.74 | 530.65 ± 6.82 | 745.64 ± 9.51 |
| ALT | 37.85 ± 1.44 | 94.60 ± 2.34 | 119.65 ± 2.93 | 179.68 ± 6.87 |

⇒ Values are expressed in Units / L.

⇒ Average of six values in each groups ± SD.

**Table 3.10 a One way ANOVA for Serum AST**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 1679685 | 3 | 559895 | 15265.17 | 9.3E-34 | 3.098393 |
| Within concentrations | 733.5588 | 20 | 36.67794 | | | |
| Total | 1680418 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 3.10 b One way ANOVA for Serum ALT**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 62244.29 | 3 | 20748.1 | 1284.149 | 4.98E-23 | 3.098393 |
| Within concentrations | 323.1416 | 20 | 16.15708 | | | |
| Total | 62567.43 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

There was a significant ($P<0.001$) increase in serum Aspartate transaminase (AST) and Alanine transaminase (ALT) on exposure to the different concentrations of WAF for three weeks when compared to control (Table 3.10 a and 3.10 b). The maximum increase in serum AST and ALT was observed in fishes exposed to 5.0ppm concentration of WAF This was followed by 3.0ppm dosed and lastly 1.5ppm treated animals. Comparison by

LSD analysis (Table 3.10 c) revealed significant (P<0.001) increase in both serum enzymes in all concentration treated groups when compared to control. LSD value at 5% level was 7.3078 and 4.8502 respectively. Also there were significant difference (P<0.001) between the different concentration treated groups when compared themselves.

**Table 3.10 c Results of LSD analysis for different WAF concentrations**

| GROUPS | PARAMETERS | | |
|---|---|---|---|
| | Blood Urea | Serum AST | Serum ALT |
| Control Vs 1.5ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| Control Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5ppm Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5ppm Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |

## 3D. DISCUSSION

Exposure of *O.mossambicus* to 1.5ppm, 3.0ppm and 5.0ppm concentrations of water-accommodated fractions of crude oil showed a steady, significant decrement in the levels of proteins in different tissues. The order of decrease of protein content in the four tissues of fish subjected to three exposures is as follows: Liver > Heart > Gill > Muscle. The decrease in proteins of different tissues suggests the existence of high protein hydrolytic activity due to the elevation of proteases as observed in the present study. From the results it is clear that when the fish was exposed to 5.0ppm concentration of water-accommodated fractions, the impact of protein

hydrolysis was greater than 3.0ppm and 1.5ppm exposures. This would lead to an increase in the free amino acid pool (Bayne *et al.*, 1981). This result stands in good agreement with the observation reported by Viarengo and Moore (1982), who observed that petroleum hydrocarbons significantly reduced the protein level and thus disrupted the normal metabolism of the digestive gland of *M.edulis*. Similar decrement in protein content was also observed when carp *Cyprinus carpio* were exposed to Shaymskaya crude oil (Mikhaylova, 1983). The amino acids may be utilized for ATP production in two different ways. They could be converted to keto acid via transaminase and then fed to the TCA cycle. Alternatively they could be channeled into gluconeogenic pathway. Oxidation in Kreb's cycle to meets the higher energy demands under petroleum hydrocarbon toxic impact. On exposure to 1.5ppm, 3.0ppm and 5.0ppm water-accommodated fractions of crude oil; it is observed that the protease activity is elevated in all the tissues, suggesting stimulation of the enzyme during toxic stress. Of the four tissues, the elevation in protease activity is highest in liver followed by heart, muscle and gill tissues suggesting that the highest toxic impact was felt on the liver tissue and the maximum increase was observed in 5.0ppm dosed animals. The uniform and consistent increase in protease activity in all the tissues of fish exposed to the three types of WAF exposures envisages protein hydrolysis, thereby deteriorating the structural integrity of the tissues. When juvenile blue crabs, *Callinecter Sapidus*, were exposed to sub lethal concentrations of water-soluble fraction of South Louisiana crude oil (0 to 2500ppb), for 21 days, they showed similar observations (Wang, 1988).

The total free amino acid content showed a significant increase in the tissues of fish exposed to different concentrations of water-accommodated fractions. The tissue specific increase in the free amino acid is as follows: Liver > Heart > Gill > Muscle. Among the four tissues investigated, liver seems to be highly affected when compared to other tissues because liver is the major site of detoxification. The increase in the free amino acid content is supported by increased proteolytic action caused by the stress of the petroleum hydrocarbons. From the results it is inferred that the increased free amino acids can be utilized for energy production (ATP) by feeding them into the TCA cycle through aminotransferase reaction. In agreement with our result Dange *et al.* (1985) also observed a similar effect in *O.mossambicus* when exposed to toluene, a water soluble fraction of crude oil.

The rate of ammonia excretion is normally closely related to the metabolic rate of an animal (Robert *et al.*,1998). In the present study a significant elevation of ammonia was observed in all the tissues of fish exposed to water-accommodated fractions and the trend is as follows: Liver > Heart > Gill > Muscle. Stickle *et al.* (1984) have studied an elevated rate of ammonia excretion in the marine snails *Thais lima* during long-term oil exposure reflecting enhanced utilization of protein reserves for maintenance and survival. In agreement with this observation, in the present study, there was an elevation in the levels of ammonia which corroborates with the increased levels of protein hydrolysis, since ammonia forms the main end product of protein catabolism (Robert *et al.*,1998).

The levels of urea in the fish exposed to 1.5ppm, 3.0ppm and 5.0ppm of water-accommodated fractions showed a steady and statistically

significant increase over the control in all the four tissues as well as in blood. The elevation of urea level remained highest under 5.0ppm WAF dosed experimental animals. The order of increase in urea level in different tissues is as follows: Liver > Heart > Gill > Muscle. The increased level of urea under petroleum hydrocarbons stress may be due to the increased degradation of nucleotides (Bayne *et al.,* 1985). Even though fish are mostly ammonotelic there are many reports on the excretion of urea by fish (Wekell and Brown, 1973).

Glutamate dehydrogenase (GDH) activity was also significantly decreased in animals exposed to different concentrations of water-accommodated fractions of crude oil. The order of decrease in glutamate dehydrogenase in tissues is as follows: Liver > Heart > Gill > Muscle. The maximum decrease in GDH activity suggests accumulation of glutamine in the tissues during petroleum hydrocarbons exposure. The accumulated glutamate may aid in meeting the energy demands under the toxic impact of crude oil, by being fed into the TCA cycle through amino transferase reactions. This is also in agreement with the earlier reports suggesting enhanced activity of aspartate amino transferase during crude oil exposure which feeds glutamate into the Kreb's cycle as its keto acid (Narvia *et al.,* 1997). An inhibition of GDH activity in gill, muscle, brain, liver and kidney of fishes exposed to toxicants has also been observed by Ghosh (1985).

Transaminases have an important role in the metabolic adjustments in response to stress. Under exposure to 1.5ppm, 3.0ppm and 5.0ppm concentrations of water-accommodated fractions, both transaminases-AST and ALT were found to be highly elevated in all the four tissues as well as in the serum; confirming the augmentation of stress as a

consequence of crude oil impact. The tissue specific increase is as follows: Liver > Muscle > Heart > Gill. The magnitude of increase in transaminase activity is directly proportional to the concentration of water-accommodated fractions of crude oil. Similar findings were observed by Narvia *et al.* (1997), when *Mytilus edulis L.* were exposed to crude oil in brackish water Since carbohydrates form the key substrates for energy metabolism, the remarkable decrease in the total carbohydrate and glycogen levels (Table 2.1 and 2.2) should necessitate enhancement of aminotransferase reactions to feed amino acid into the glycolytic and TCA cycle or directed into the gluconeogenic pathway. It has been reported that crude oil causes enhancement of AST and ALT in different tissues of *O.mossambicus* exposed to 25ppm and 50ppm of toluene, a major water soluble and toxic component of various crude oils, for 10 weeks (Dange *et al.*, 1985). The other possible reason for the increased level of AST and ALT are may be a stress induced increase in the circulating levels of glucocorticoid hormones (e.g. cortisol). Similar type of observation was also observed by Janice *et al.* (1979), when American oysters and brown shrimps were exposed to chronic exposure to crude oil.

Thus it may be summarized that the nitrogen metabolism of *O.mossambicus* dosed with water-accommodated fractions of crude oil was significantly affected. The elevation in proteases, free amino acids, ammonia, urea, glutamine, AST and ALT with corresponding decrease in protein levels and GDH in the selected tissues showed the effective operation of regulatory mechanisms in fish exposed to water-accommodated fractions of crude oil.

*Chapter* 4

# Effects Of Petroleum Hydrocarbons On Lipid Peroxidation

## 4A. INTRODUCTION

A fundamental aspect of aerobic life is the potentially dangerous sequelae that might be engendered when antioxidant defenses are overcome by prooxidant forces. This action, commonly referred to as oxidative stress, now appears to be the basis of a wide variety of physiological aberrations in aquatic organisms (Kappus, 1986). As the aquatic environment is a major sink for petroleum hydrocarbons, the elucidation of biochemical responses to this contaminant by aquatic animals becomes increasingly important. Most recent evidence indicates that the health of aquatic organisms might be linked to the deleterious consequences of oxygen utilization (Di Giulio *et al.*, 1989, Livingstone, 2001). Of particular concern are processes by which petroleum hydrocarbons may enhance oxidative stress in aquatic organisms. During its metabolism, quinones may be formed which have the potential to produce reactive oxygen species by redox cycling (Lemaire and Livingstone, 1997).

### 4A.1 SOURCES OF OXYRADICALS

Aquatic life is dependent upon molecular oxygen ($O_2$) for the provision of energy through the coupling of oxidation to energy transfer via the phosphorylation of ADP This process is handled by the mitochondrial electron transport chain in which oxygen undergoes a concerted four electron reduction to water (Sole *et al.*, 1996). As a consequence of concerted reduction, partially reduced oxygen results and various reactive oxygen species (oxyradicals) are produced (Figure 4.1)

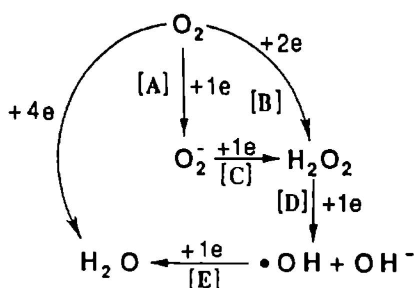
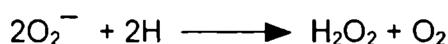



**Figure 4.1 Electron-transfer reactions during oxygen reduction metabolism.**

Four electrons are required for the complete reduction of molecular oxygen to water during aerobic respiration. Reduction by 1electron [A] produces superoxide anion radical; hydrogen peroxide is formed by direct 2 electron reduction [B] or by concerted 1 electron steps [A] and [C]; the potently oxidizing hydroxyl radical is generated via electron reduction of hydrogen peroxide [D]; 1 electron reduction of hydroxyl radical by superoxide dismutase and of hydrogen peroxide by catalase that serve to prevent the formation of radical intermediates of oxygen reduction metabolism (Gary and Richard (1991)).

Oxyradicals have been implicated in oxidative tissue damage and free radical pathology (Kappus and Sies, 1981; Halliwell and Gutteridge, 1984). Figure 1 indicates that one, two and three electron-reduced intermediates are possible, each of which potentially is deleterious to cells. Univalent reduction of oxygen produces the superoxide anion radical, $O_2^-$ which can dismutate to hydrogen peroxide, $H_2O_2$.

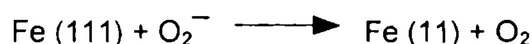


$$2O_2^- + 2H \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide can serve as a precursor of the hydroxyl radical, OH, via the Haber-Weiss reaction.

$$O_2^{-} + H_2O_2 \longrightarrow O_2 + OH + OH^{-}$$

Although thermodynamically favorable, the reaction is kinetically slow (Halliwell, 1978); thus, transition metals particularly iron, are generally required as catalyst for significant ˙OH production, via Fenton reaction (Walling, 1975).



$$Fe\,(111) + O_2^{-} \longrightarrow Fe\,(11) + O_2$$

$$Fe\,(11) + H_2O_2 \longrightarrow Fe\,(111) + OH + OH^{-}$$

The hydroxyl radical is among the most potent oxidants known, capable of reacting kinetically indiscriminately with virtually all organic chemicals, including critical cellular macromolecules, possibly leading to protein degradation and enzyme inactivation, lipid peroxidation, DNA damage and ultimately, cell death (Viarengo *et al.,* 1991). The process of oxyradicals does not preclude much deleterious disease states as the degree of physiological damage is limited by the presence of an armamentarium of antioxidant defense mechanisms in fish (Diguiseppi and Fridovich, 1984). Thus, oxidative damage reflects an imbalance between the production of oxidants and the removal or scavenging of those oxidants. Specially adopted enzymes, thus protect aquatic aerobic organisms from the ravages of oxyradicals (Cajaraville *et al.*, 1997).

## 4A.2 LIPID PEROXIDATION

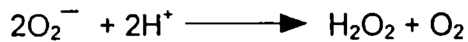
Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated lipids. Lipid peroxidation arising from the direct reaction of free radicals with lipids is considered as prevalent, important feature of cellular injury brought about by free radical attack. This progresses by three operationally defined processes: initiation, propagation and termination. The initiation phase of peroxidation usually proceeds with the formation of conjugated diene bonds generated by abstraction of hydrogen atoms from the methylene group of poly unsaturated lipids. Propagation of lipid peroxidation relies on the interaction of molecular oxygen with carbon centered free radicals to form lipid hydroperoxides. These lipid hydroperoxides can form free radicals to propagate the lipid peroxidation. Lipid peroxidation usually affects membrane lipids. In addition to the self destructive nature of membrane lipid peroxidation arising from free radical generation by other membranes, lipid peroxidation is a major source of other cytotoxic products such as malondialdehyde produced from the decomposition of lipid hydroperoxides. Malondialdehyde reacts with nitrogenous bases of DNA to form DNA adducts (Fujimoto *et al.*, 1984).

## 4A.3 ANTIOXIDANT DEFENSE AGAINST OXYRADICALS

Superoxide dismutase (SOD), catalase (CAT) and peroxidases constitute mutually supportive team of defense against oxyradicals. While SOD lowers the steady state level of $O_2^-$ catalase and peroxidases do the same for $H_2O_2$.

**a. Superoxide dismutase (SOD)**

SOD catalyses 1ē dismutation of superoxide anion free radicals to hydrogen peroxide and oxygen (Mac Millan-Crow *et al.*, 1998).



$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

SOD is a metalloprotein found in both prokaryotic and eukaryotic cells. The iron containing (Fe-SOD) and the manganese containing (Mn-SOD) enzyme are characteristic of prokaryotes (Sheehan, 1999). In eukaryotic cells the predominant forms are the copper containing enzyme and the zinc containing enzyme located in the cytosol. The second type is the manganese containing SOD found in mitochondrial matrix which contributes up to 60% to total tissue activity (Radi *et al.*, 1985).

**b. Catalase (CAT)**

Catalase detoxifies hydrogen peroxide to oxygen and water and thus protects the cell from oxidative damage by $H_2O_2$ and $^{\cdot}OH$

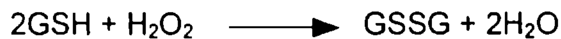
$$2H_2O_2 \longrightarrow 2\,H_2O + O_2$$

This enzyme primarily occurs in peroxisomes and it is an enzyme of high molecular weight containing a porphyrin nucleus.

**c. Glutathione peroxidase (GPX)**

Glutathione peroxidase is the most important peroxidase for the detoxification of hydroperoxides. It catalyzes the glutathione-dependent reduction of hydroperoxides and of hydrogen peroxide. GPX are localized in the peroxisomes of fish liver cells (Orbea *et al.*, 2000).

$$2GSH + ROOH \longrightarrow GSSG + ROH + H_2O$$

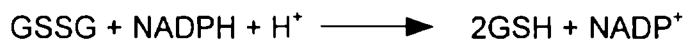


$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

It is characterized by its content of functional selenium and by its ability to reduce hydrogen peroxide and a large number of organic0020hydroperoxides. Glutathione peroxidase and other glutathione metabolizing enzyme activities are strongly dependent on tissue, species and developmental stage (Aceto *et al.*, 1994). Fish being more susceptible to oxidative damage have generally higher glutathione peroxidase activities (Hasspieler *et al.*, 1994) than other enzymes involved in glutathione metabolism.

**d. Glutathione reductase (GR)**

In cytosolic and mitochondrial compartments the oxidized glutathione (GSSG) is reduced at the expense of NADPH by the ubiquitous flavin containing enzyme GR (Schirmer and Siegel, 1989).



$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

NADPH is recycled by glucose-6-phosphate dehydrogenase via pentose phosphate pathway of glucose oxidation.

**e. Glutathione (GSH)**

GSH is a tripeptide and is gamma glutamyl cysteinyl glycine. Glutathione is the most abundant thiol in most tissues. Its function is two-fold: it is an antioxidant, which scavenges $O_2^-$ and a cofactor for enzymatic reactions like the conjugation of xenobiotics by glutathione-S-transferase (Gallagher *et al.*, 1992). When glutathione scavenges a radical, a thiol radical

is formed, which undergoes dimerisation to oxidized glutathione disulfide, or mixed disulfides with proteins are formed. Both glutathione disulfide and mixed disulfides with proteins are indicators of increased reactive oxygen species, and both are reduced by glutathione reductase, which restores the original glutathione concentrations at the expense of cellular NADPH (Otto and Moon, 1995). Glutathione is necessary for glutathione peroxidase, which detoxifies organic and inorganic peroxides, and for glutathione-S-transferase, which are involved in the conjugation and excretion of xenobiotics. The whole reaction of oxygen metabolism in combination with glutathione metabolism is represented in Figure 4.2.

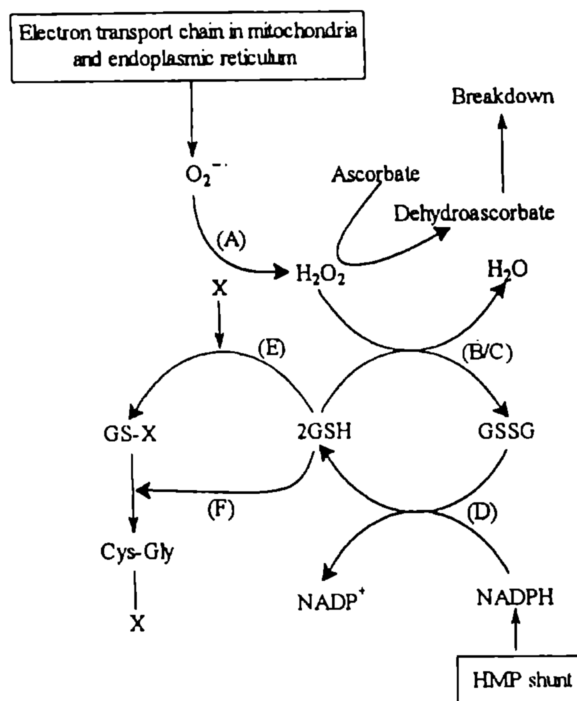



**Figure 4.2 Free radical pathway in combination with glutathione metabolism is given below.**

(A) Superoxide dismutase (B) Catalase (C) Glutathione peroxidase
(D) Glutathione reductase (E) Glutathione-S-transferase (F) γ-Glutamyl transpeptidase

### (f) Conjugated dienes (CD) and malondialdehyde (MDA)

The quantification of oxidative stress is complicated by the short half-lives of both free radicals and many of the products initially produced by free radical attack on electron-rich substrates such as polyunsaturated fatty acids (PUFAs) (Esterbauer, 1996). Consequently, the detection of oxidative stress has relied largely on the quantification of compounds such as conjugated dienes, hydroperoxides as well as malondialdehyde (MDA), which are formed by degradation of initial products of free radical attack (Janero, 1990). The reaction of MDA with thiobarbituric acid (TBA) is one of the most widely used estimators of oxidative stress (Liu *et al.*, 1997). Thus the levels of which can serve as an indicator of cell membrane damage by petroleum hydrocarbons.

The primary objective of this study was to investigate the peroxidative changes in *O.mossambicus* exposed to 1.5ppm, 3.0ppm and 5.0ppm concentrations of water-accommodated fractions of crude oil for 3 weeks.

## 4B. MATERIALS AND METHODS

Collection, acclimation of fish, WAF preparation and method of WAF dosing in *O.mossambicus* (15 ± 3) were the same as that described in the Chapter 2B.

### 4B.1 METHODS USED FOR THE BIOCHEMICAL ANALYSIS

The following are the parameters studied under lipid peroxidation.

**a. Estimation of tissue superoxide dismutase (SOD) (E.C.1.15.1.1)**

Superoxide dismutase in different tissues was determined using the method of Kakkar *et al.* (1984).

**Reagents**

0.33 M sucrose, n-butanol, 0.052 M sodium pyro-phosphate buffer (pH 8.3), 0.0025 M Tris-HCl buffer (pH 7.4), 186 μM phenazine methosulphate (PMS), 300 μM Nitro blue tetrazolium (NBT), 780 μ M NADH and glacial acetic acid.

**Procedure**

Weighed samples of tissues were homogenized in 0.33 M sucrose and subjected to differential centrifugation under cold conditions to obtain the cytosol fraction. Before estimating the activity, an initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and this fraction was then dialysed against 0.0025 M Tris-HCl buffer (pH 7.4). The supernatant was used as the enzyme source. Assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3 ml of NBT, 1.3 ml of distilled water and 0.1 ml of the enzyme source. The tubes were kept at 30°C for one minute and then 0.2 ml of NADH were added and incubated at 30°C for 90 sec and the reaction was stopped by the addition of 1 ml of glacial acetic acid. Reaction mixture was shaken vigorously with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. The upper butanol layer was removed. Absorbance of the

chromogen in butanol was measured at 560 nm against n-butanol blank. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in one minute under the assay conditions and specific activity is expressed as units / mg protein.

**b. Estimation of Catalase (CAT) (E.C.1.11.1.6)**

Catalase level in different tissues was determined using the method of Maehly and Chance, 1955.

**Reagents**

0.01 M phosphate buffer (pH 7.0), 30 mM $H_2O_2$.

**Procedure**

The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer, 30 mM hydrogen peroxide and the enzyme extract prepared by homogenizing the tissue in phosphate buffer and centrifuging at 5000 rpm. Specific activity was expressed as International Units / mg protein. 1 IU = change in absorbance / min / extinction coefficient (0.021).

**c. Estimation of Glutathione peroxidase (GPX) (E.C. 1.11.1.9)**

Glutathione peroxidase in different tissues was estimated by the method of Rotruck, 1973.

**Reagents**

0.4 M Tris buffer (pH 7.0), 10mM sodium azide solution, 10% Trichloro acetic acid (TCA), 0.4 mM Ethylene diamine tetra acetic acid (EDTA), 0.2 mM Hydrogen peroxide ($H_2O_2$), 2 mM glutathione solution (GSH).

**Procedure**

Weighed sample of different tissues was homogenized in a known volume of Tris buffer To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml sodium azide and 0.5 ml tissue homogenate were added and mixed well. To this mixture 0.2 ml of GSH followed by 0.1 ml $H_2O_2$ solution were added. The contents were mixed and incubated at 37°C for 10 minutes along with a control containing all reagents except tissue homogenate. After 10 minutes the reaction was arrested by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and the supernatant was assayed for GSH. The values are expressed as µg of GSH / min / mg protein.

**d. Estimation of Glutathione reductase (GR) (E.C. 1.6.4.2)**

Glutathione reductase was estimated by the method of Bergmeyer, 1974.

**Reagents**

0.067 M phosphate buffer (pH 6.6), 0.06% NADPH, 15mM EDTA, 1.15% glutathione.

**Procedure**

Weighed sample of different tissues was homogenized in a known volume of phosphate buffer. The decrease in absorbance containing glutathione (GSSG) (oxidized), NADPH, EDTA and phosphate buffer (pH 6.6) was noted for 3-5 minutes at 340 nm in a UV-visible spectrophotometer. The controls were run with water instead of GSSG. Enzyme activity was expressed as units / mg protein. One unit was defined as the change in absorbance / minute.

**e. Estimation of Glutathione-S-transferase (GST) (E.C. 2.5.1.18)**

Glutathione-S-transferase in different tissues was determined using the method of Beutler *et al.* (1986).

**Reagents**

0.5 M phosphate buffer (pH 6.5), 25 mM of 1-chloro-2, 4-dinitro benzene (CDNB) in 95% ethanol, 20 mM glutathione (GSH).

**Procedure**

Different tissues were homogenized in 0.5M phosphate buffer The reaction mixture containing 200 µl phosphate buffer, 20 µl CDNB and 730 µl distilled water were taken in the control tube and 200 µl phosphate buffer, 20 µl CDNB and 680 µl distilled water taken in the test sample tubes. Then the tubes were incubated at 37°C for 10 minutes. After the incubation added 50 µl of GSH in both set of tubes. After mixing well, added 50 µl of tissue extract in the test sample tube. Increase in absorbance was noted at

340 nm for 5 minutes in a quartz cuvette of 1 cm length path in a UV- visible spectrophotometer. Values are expressed in nmoles of CDNB complexed / min / mg protein. The extinction coefficient between CDNB-GSH conjugate and CDNB is 9.6 $mM^{-1}$ $cm^{-1}$

**f. Estimation of tissue glutathione**

Glutathione level in different tissues was determined using the method of Patterson and Lazarov, 1955.

**Reagents**

0.1 M alloxan, 0.5 M (pH 7.5) phosphate buffer, 0.5 N NaOH and 1 N NaOH.

**Procedure**

Weighed sample of tissues were homogenized in 0.5M phosphate buffer. The incubation mixture contained 50 µl tissue extract, 50 µl alloxan, 50 µl phosphate buffer and 50 µl 0.5 N NaOH. The mixture was incubated for 6 minutes at 25°C. The reaction was arrested by the addition of 50 µl of 1 N NaOH. Absorbance was noted at 305 nm in a quartz cuvette of 1 cm length path in a UV-visible spectrophotometer. A control tube was maintained with phosphate buffer instead of extract. The values are expressed in mg / 100 g tissue.

**g. Estimation of Conjugated dienes (CD)**

The concentration of conjugated dienes was estimated according to the method of Retnagal and Ghoshal, 1966.

**Procedure**

Membrane lipids were extracted and evaporated to dryness as described for the iodometric assay for hydroperoxides. The lipid residue was dissolved in 1.5ppm ml of cyclohexane and the absorbance at 233 nm was determined against a cyclohexane blank. Molar extinction coefficient of conjugated dienes is $2.52 \times 10^4$ $M^{-1}$ $cm^{-1}$

**h. Estimation of Hydroperoxides**

Hydroperoxides was estimated by method of Mair and Hall, 1977

**Reagents**

Potassium iodide (KI), 0.5% cadmium acetate.

**Procedure**

1 ml of the tissue homogenate of the different tissues was mixed thoroughly with 5 ml of chloroform: methanol (2:1) followed by centrifugation at 1000 g for 5 minutes to separate the phases. 3 ml of the lower chloroform layer was recovered using a syringe and placed in a test tube and dried in a 45°C 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 centrifuged at 1000 g 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 is $1.73 \times 10^4$ $M^{-1}$ $cm^{-1}$

### i. Estimation of Malondialdehyde

Malondialdehyde was estimated by the method of Niehaus and Samuelson, 1968.

**Reagents**

TCA-TBA-HCl reagent 15% (w/v) Trichloro acetic acid, 0.375% (w/v) Thio-barbituric acid (TBA) in 0.25 N HCl, 0.1 M Tris-HCl buffer (pH 7.5).

**Procedure**

The tissue homogenate of different tissues were prepared in Tris-HCl buffer and was combined with thiobarbituric acid and mixed thoroughly and heated for 15 minutes in a boiling water bath. It was then cooled and centrifuged for 10 minutes at 600 g. 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 $1.56 \times 10^{-5}$ / $M^{-1}$ / $cm^{-1}$ The values are expressed as millimoles / 100 g wet wt. tissue.

Protein was estimated by the method of Lowry *et al.* (1951).

## 4C. RESULTS

Effect of different concentrations of WAF exposure for three weeks on the antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), detoxifying enzymes like Glutathione reductase (GR), Glutathione-S-transferase (GST) and Glutathione as well as lipid peroxidation products such as conjugated dienes (CD), hydroperoxides

and malondialdehyde are studied in the liver, gill, heart and muscle tissues of *O. mossambicus* and the results were given in tables 4.1 to 4.9 and in figures 4.3 to 4.11. The statistical significance of the results was tested by ANOVA followed by LSD (Least Significance Difference) analysis.

**Table 4.1 Effect of different concentrations of WAF exposure on the Superoxide dismutase (SOD) activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 11.73 ± 1.47 | 12.11 ± 1.92 | 11.25 ± 1.13 | 4.34 ± 0.89 |
| 1.5ppm | 15.28 ± 1.82 | 18.35 ± 2.11 | 23.37 ± 2.67 | 4.68 ± 0.98 |
| 3.0ppm | 26.44 ± 2.96 | 24.77± 3.25 | 28.71 ± 3.05 | 7.95 ± 1.27 |
| 5.0ppm | 10.32 ± 1.12 | 19.25 ± 1.98 | 20.45 ± 3.12 | 7.02 ± 1.39 |

⇒ Values are expressed as units / mg protein.
One unit is defined as the amount of enzyme which gives 50% inhibition of the formazan formation / minute.

⇒ Average of six values in each group ± SD.

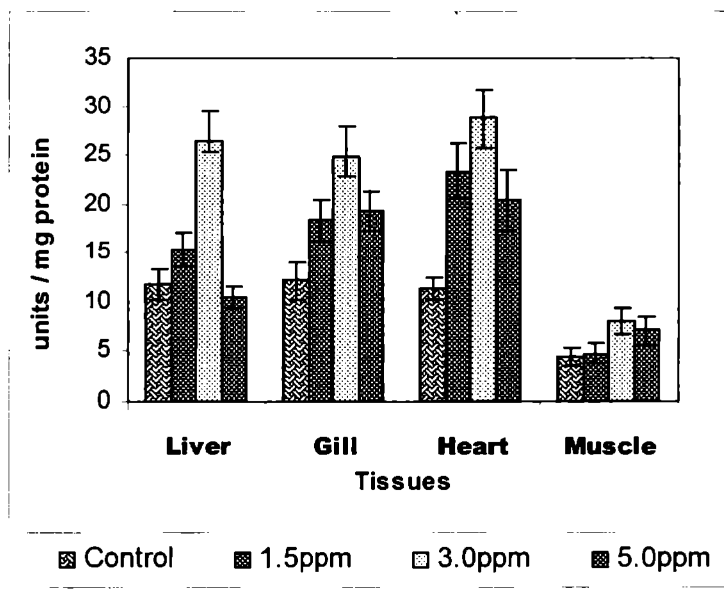



**Figure 4.3 Activity of SOD in the various tissues exposed to different concentrations of WAF**

**Table 4.1 a Two-Factor ANOVA for SOD activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 300.1574 | 3 | 100.0525 | 8.334247 | 0.00578 | 3.862539 |
| Between tissues | 519.4919 | 3 | 173.164 | 14.42434 | 0.000874 | 3.862539 |
| Error | 108.0448 | 9 | 12.00498 | | | |
| Total | 927.6942 | 15 | | | | |

SS-sum of squares, df-degrees of freedom, MS-mean of squares

Two-Factor ANOVA (Table 4.1a) and further comparisons by LSD analysis (Table 4.5 b) revealed that SOD activity was significantly increased in 1.5ppm and 3.0ppm WAF treated groups when compared to the control. However there was no significant increase in 5.0ppm WAF treated group when compared to control. LSD value at 5% level was 5.540. Comparison between the different WAF concentrations revealed that there was a significant increase between 3.0ppm and 1.5ppm WAF treated groups as well as 5.0ppm and 3.0ppm WAF treated groups with the exception of 5.0ppm and 1.5ppm WAF treated groups. Further comparison using LSD analysis (Table 4.5c) revealed that significant difference was noted between different tissues with the exception of liver and gill, liver and heart, and heart and gill.

**Table 4.2 Effect of different concentrations of WAF exposure on Catalase (CAT) activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 13.03 ± 1.45 | 16.78 ± 1.61 | 11.28 ± 1.58 | 4.25 ± 1.02 |
| 1.5ppm | 29.36 ± 2.55 | 59.11 ± 4.11 | 35.61 ± 3.42 | 9.23 ± 1.54 |
| 3.0ppm | 41.88 ± 3.58 | 69.23 ± 4.17 | 36.39 ± 2.27 | 16.15 ± 2.19 |
| 5.0ppm | 18.27 ± 2.62 | 28.67 ± 3.46 | 25.26 ± 2.19 | 10.11 ± 1.03 |

⇒ One IU = change in absorbance at 230 nm / min, Extinction coefficient =0.021

⇒ Average of six values in each groups ± SD.

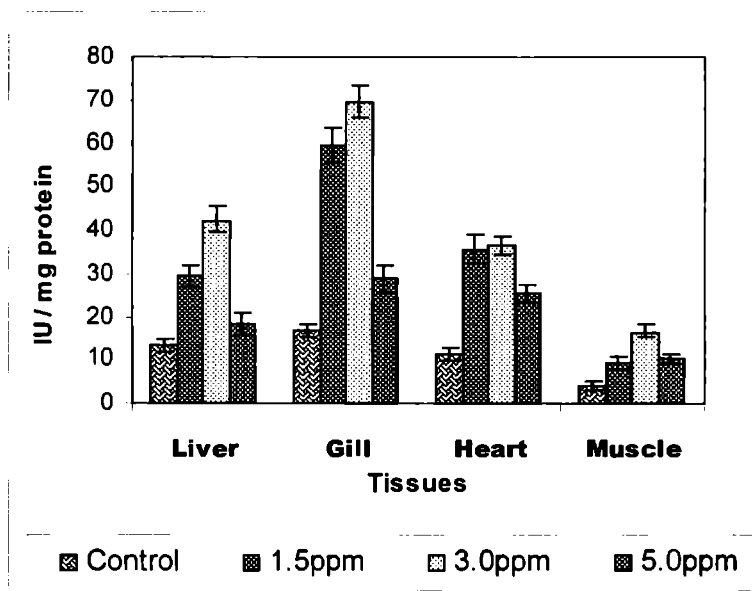



**Figure 4.4 Activity of catalase in the various tissues exposed to different concentrations of WAF**

**Table 4.2 a Two-Factor ANOVA for Catalase**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 2077.529 | 3 | 692.5098 | 8.458912 | 0.005511 | 3.862539 |
| Between tissues | 2251.05 | 3 | 750.3502 | 9.165425 | 0.004243 | 3.862539 |
| Error | 736.8073 | 9 | 81.8674 | | | |
| Total | 5065.387 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two way analysis of variance (Table 4.2 a) and subsequent comparisons by LSD analysis (Tables 4.5 b) revealed that there was a significant increase ($P<0.001$) in catalase activity in 1.5ppm and 3.0ppm WAF dosed experimental animals when compared to the control group. Comparison between control and 5.0ppm WAF treated group did not reveal any significant difference. LSD value at 5% level was 14.47.Comparisons between the concentrations showed that there was a significant difference

between 3.0ppm and 1.5ppm WAF treated group as well as 5.0ppm and 3.0ppm treated groups with the exception of 5.0ppm and 1.5ppm treated groups. LSD analysis (Table 4.5c) also revealed that there was a significant difference between all the tissues except between liver and heart.

**Table 4.3 Effect of different concentrations of WAF exposure on the activity of Glutathione peroxidase (GPX) in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 8.74 ± 1.23 | 12.85 ± 2.07 | 11.75 ± 1.32 | 5.85 ± 0.99 |
| 1.5ppm | 28.71 ± 3.54 | 45.23 ± 4.30 | 21.95 ± 2.47 | 9.05 ± 1.17 |
| 3.0ppm | 27.45 ± 3.28 | 41.85 ± 3.37 | 22.94 ± 3.00 | 10.74 ± 1.28 |
| 5.0ppm | 13.67 ± 1.82 | 10.63 ± 1.20 | 16.52 ± 1.96 | 9.85 ± 1.08 |

⇒ Values are expressed as μ g of GSH / min / mg protein.

⇒ Average of six values in each groups ± SD.

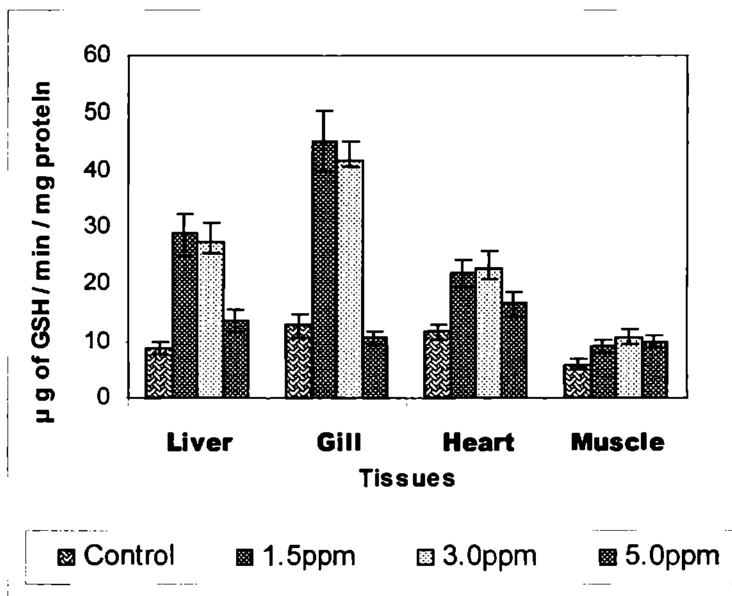



**Figure 4.5 Activity of glutathione peroxidase in the various tissues exposed to different concentrations of WAF**

**Table 4.3 a Two-Factor ANOVA for Glutathione peroxidase**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 888.0892 | 3 | 296.0297 | 5.08863 | 0.024861 | 3.862539 |
| Between tissues | 710.113 | 3 | 236.7043 | 4.06885 | 0.044119 | 3.862539 |
| Error | 723.5717 | 9 | 58.1746 | | | |
| Total | 2121.774 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 4.3 a) and further LSD analysis (Tables 4.5 b) revealed that there was a significant increase ($P<0.05$) in GPX activity between 1.5ppm and control, as well as 3.0ppm and control group. However, 5.0ppm of WAF induced insignificant activation of the enzyme when compared to the control. LSD value at 5% level was 12.20. Comparison between concentrations revealed that there was a significant change between all the concentrations except between 3.0ppm and 1.5ppm WAF treated groups. LSD analysis (Table 4.5c) also revealed that there was a significant difference between liver and muscle, and also between gill and muscle. All other comparison between tissues showed insignificant change.

**Table 4.4 Effect of different concentrations of WAF exposure on the Glutathione Reductase (GR) x $10^{-3}$ activity in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 5.13 ± 0.71 | 4.15 ± 0.91 | 4.11 ± 0.53 | 2.62 ± 0.35 |
| 1.5ppm | 9.09 ± 0.83 | 5.09 ± 0.92 | 5.36 ± 0.77 | 3.85 ± 0.29 |
| 3.0ppm | 10.89 ± 1.02 | 10.48 ± 1.21 | 9.75 ± 0.99 | 3.97 ± 0.18 |
| 5.0ppm | 3.85 ± 0.56 | 2.21 ± 0.58 | 5.14 ± 0.87 | 2.13 ± 0.11 |

⇒ Values are expressed as units / mg protein.
Unit is defined as the change in absorbance at 340 nm / min / mg protein.

⇒ Average of six values in each groups ± SD.

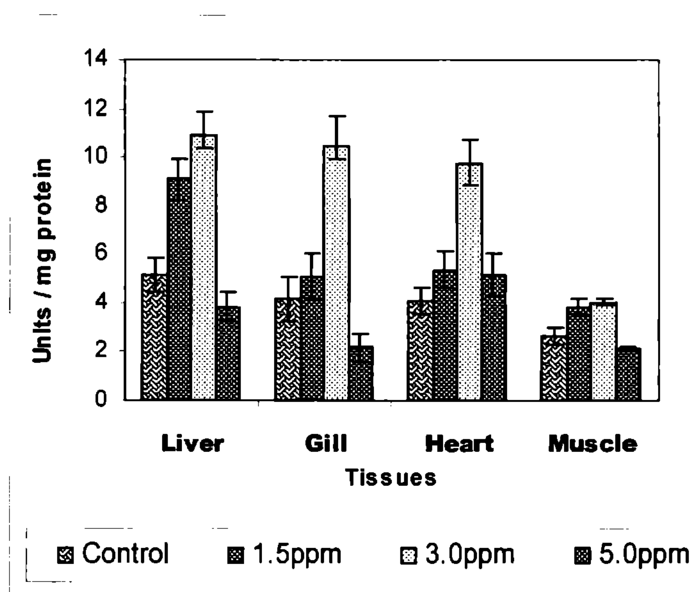



**Figure 4.6 Activity of GR in the various tissues exposed to different concentrations of WAF**

**Table 4.4 a Two-Factor ANOVA for GR activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 71.01722 | 3 | 23.67241 | 10.55035 | 0.002646 | 3.862539 |
| Between tissues | 35.41952 | 3 | 11.80651 | 5.261938 | 0.022703 | 3.862539 |
| Error | 20.19381 | 9 | 2.243756 | | | |
| Total | 126.6305 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 4.4 a) and subsequent LSD analysis (Table 4.5 b) revealed that there was a significant increase (P<0.05) in glutathione reductase activity only between control and 3.0ppm WAF dosed experimental animals. Whereas comparison between control and 1.5ppm treated groups as well as control and 5.0ppm treated groups did not reveal any significant change. Further comparison between concentrations using LSD analysis revealed that there was a significant difference between 5.0ppm treated group with that of 1.5ppm and 3.0ppm treated ones, with the

exception between 3.0ppm and 1.5ppm WAF dosed fish. LSD value at 5% level was 2.39. LSD analysis (Table 4.5 c) also revealed that there was a significant difference between tissues with the exception of liver and heart, as well as gill and heart.

**Table 4.5 Effect of different concentrations of WAF exposure on the activity of Glutathione-S-transferase (GST) in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 35.65 ± 3.74 | 28.45 ± 3.44 | 19.74 ± 2.08 | 7.31 ± 1.33 |
| 1.5ppm | 60.28 ± 5.50 | 37.28 ± 2.75 | 28.62 ± 3.19 | 10.66 ± 1.16 |
| 3.0ppm | 71.36 ± 6.43 | 58.31 ± 4.45 | 44.35 ± 5.21 | 22.75 ± 2.32 |
| 5.0ppm | 31.48 ± 4.24 | 20.43 ± 1.21 | 25.62 ± 2.77 | 11.31 ± 1.28 |

⇒ Values are expressed in n moles of CDNB complexed / min / mg protein.

⇒ Average of six values in each groups ± SD.

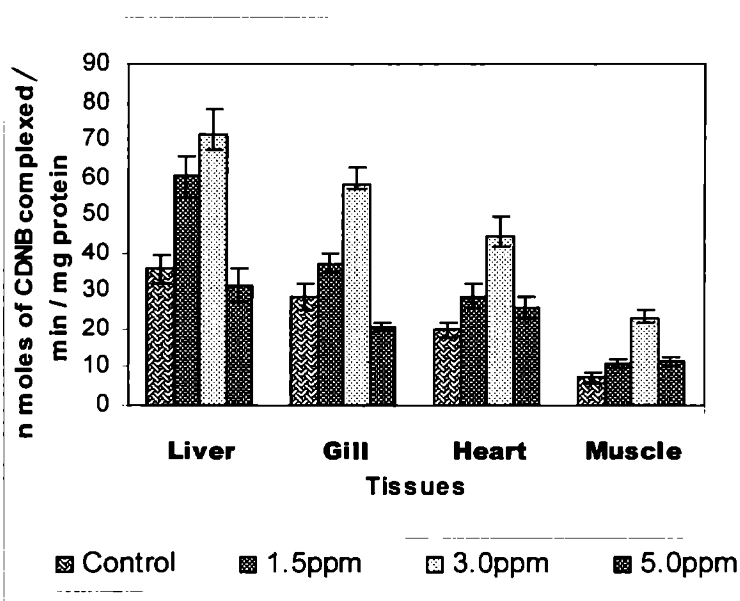



**Figure 4.7 Activity of GST in the various tissues exposed to different concentrations of WAF**

**Table 4.5 a Two-Factor ANOVA for GST**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 1924.562 | 3 | 641.5206 | 12.77365 | 0.001356 | 3.862539 |
| Between tissues | 2785.991 | 3 | 928.6636 | 18.49111 | 0.000345 | 3.862539 |
| Error | 451.9994 | 9 | 50.22216 | | | |
| Total | 5162.552 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 4.5 a) and further comparisons by LSD analysis (Table 4.5 b) revealed that there was a significant increase in GST between control and 1.5ppm WAF treated groups (P<0.05) as well as between control and 3.0ppm (P<0.001) WAF dosed group. However, there was no significant difference between control and 5.0ppm treated group. LSD value at 5% level was 11.33. Further comparisons by LSD analysis (Table 4.5b) revealed that there was a significant difference between different concentrations also. Comparisons between tissues (Table 4.5c) revealed that there was a significant change, with the exception of heart and gill.

**Table 4.5 b Results of LSD analysis for different WAF concentrations**

| GROUPS | PARAMETERS | | | | |
|---|---|---|---|---|---|
| | SOD | CAT | GPX | GR | GST |
| Control Vs 1.5ppm | P < 0.05 | P < 0.001 | P < 0.05 | NS | P < 0.05 |
| Control Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.05 | P < 0.001 | P < 0.001 |
| Control Vs 5.0ppm | NS | NS | NS | NS | NS |
| 3.0ppm Vs 1.5ppm | P < 0.05 | P < 0.05 | NS | P < 0.001 | P < 0.05 |
| 5.0ppm Vs 1.5ppm | NS | NS | P < 0.05 | P < 0.001 | P < 0.05 |
| 5.0ppm Vs 3.0ppm | P < 0.05 | P < 0.05 | P < 0.01 | P < 0.001 | P < 0.001 |

NS - Not significant

**Table 4.5 c Results of LSD analysis for different tissues.**

| GROUPS | PARAMETERS | | | | |
|---|---|---|---|---|---|
| | SOD | CAT | GPX | GR | GST |
| Liver Vs Gill | NS | P < 0.15 | NS | NS | P < 0.05 |
| Liver Vs Heart | NS | NS | NS | NS | P < 0.001 |
| Liver Vs Muscle | P < 0.001 | P < 0.01 | NS | P < 0.001 | P < 0.001 |
| Heart Vs Gill | NS | P < 0.05 | NS | NS | NS |
| Muscle Vs Gill | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| Muscle Vs Heart | P < 0.001 | P < 0.05 | NS | P < 0.001 | P < 0.001 |

NS Not significant
SOD Superoxide dismutase
CAT Catalase
GPX Glutathione peroxidase
GR Glutathione reductase
GST Glutathione-S-transferase

**Table 4.6 Effect of different concentrations of WAF exposure on the level of Glutathione (GSH) in different tissues of *O.mossambicus*.**

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 1810 ±65.07 | 1622 ± 84.09 | 995 ± 40.28 | 705 ± 38.45 |
| 1.5ppm | 2222 ± 73.18 | 1893 ± 74.14 | 1198 ± 65.92 | 913 ± 40.32 |
| 3.0ppm | 2375 ± 95.17 | 2115 ± 100.83 | 1685 ± 53.24 | 928 ± 39.67 |
| 5.0ppm | 1624 ± 50.69 | 1412 ± 92.67 | 1015 ± 67.25 | 900 ± 30.29 |

⇒ Values are expressed as n moles / 100g wet tissues.

⇒ Average of six values in each group ± SD.

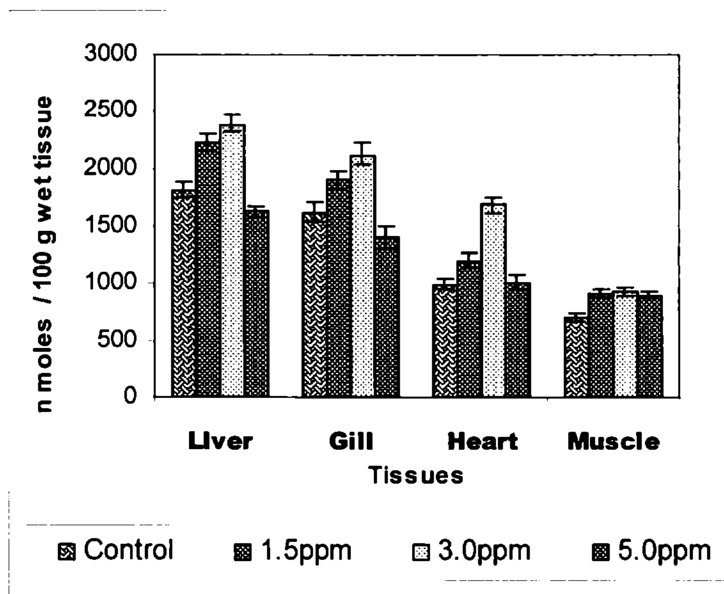



**Figure 4.8 Levels of Glutathione content in the various tissues exposed to different concentrations of WAF**

**Table 4.6 a Two-Factor ANOVA for Glutathione**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 758768 | 3 | 252922 | 9.702798 | 0.003512 | 3.862539 |
| Between tissues | 3218164 | 3 | 1072721 | 41.15246 | 1.4E-05 | 3.862539 |
| Error | 234603 | 9 | 26067 | | | |
| Total | 4211535 | 15 | | | | |

SS-sum of squares, df-degrees of freedom, MS-mean of squares.

Two-Factor ANOVA (Table 4.6 a) and subsequent LSD analysis (Table 4.9 b) revealed that there was a significant increase ($P<0.05$) in glutathione level between control and 1.5ppm as well as control and 3.0ppm WAF treated groups. Comparison between control and 5.0ppm treated group did not show any significant difference. LSD value at 5% level was 258. Further comparisons between concentrations revealed that 5.0ppm and 1.5ppm as well as 5.0ppm and 3.0ppm treated groups showed significant

difference at 5% level whereas 3.0ppm and 1.5ppm treated groups did not show any significant change. Subsequent LSD analysis also showed that (Table 4.9 c) there was an overall significant difference (P<0.001) between different tissues. The liver tissue recorded maximum increase followed by gill, heart and muscle tissues.

**Table 4.7 Effect of different concentrations of WAF exposure on the level of Conjugated Dienes (CD) in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 56.29 ± 7.67 | 31.54 ± 4.62 | 40.63 ± 6.14 | 20.45 ± 3.78 |
| 1.5ppm | 61.28 ±10.13 | 34.49 ± 5.14 | 44.52 ± 8.43 | 23.87 ± 5.43 |
| 3.0ppm | 70.39 ± 8.19 | 46.85 ± 6.17 | 49.85 ± 7.38 | 28.00 ± 4.12 |
| 5.0ppm | 92.85 ±11.26 | 51.00 ± 7.42 | 64.01 ± 9.45 | 42.56 ± 6.14 |

⇒ Values are expressed as m moles / 100 g wet tissue.

⇒ Average of six values in each groups ± SD.

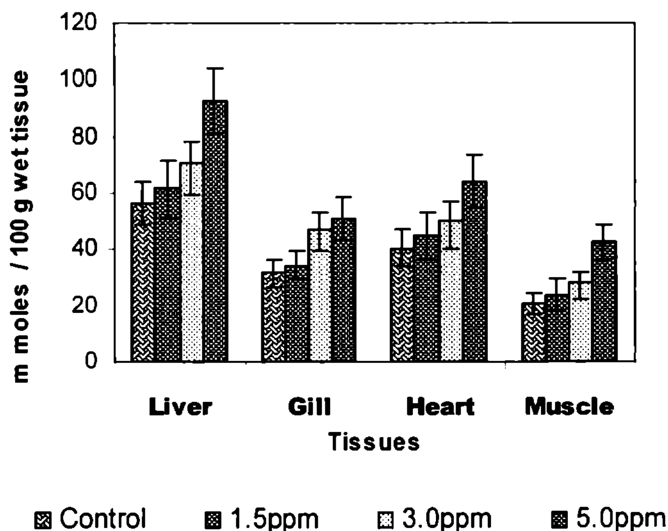



**Figure 4.9 Activity of conjugated dienes in the various tissues exposed to different concentrations of WAF**

**Table 4.7 a Two-Factor ANOVA for Conjugated Dienes**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 1508.019 | 3 | 502.6728 | 31.84846 | 4.02E-05 | 3.862539 |
| Between tissues | 3663.1 | 3 | 1221.033 | 77.36251 | 9.56E-07 | 3.862539 |
| Error | 142.0494 | 9 | 15.78327 | | | |
| Total | 5313.168 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two Factor ANOVA (Table 4.7 a) and a subsequent comparisons by LSD analysis (Tables 4.9 b ) showed a significant increase in conjugated dienes between control and 3.0ppm ($P<0.05$) as well as control and 5.0ppm ($P<0.001$) WAF treated groups. However, there was no significant difference was observed between control and 1.5ppm treated group. LSD value at 5% level was 6.35. A comparison between concentrations revealed a significant change. Similarly there was a significant difference (Table 4.9 c) between tissues. Conjugated diene level was maximum in hepatic tissue followed by gill, heart and muscle tissues.

**Table 4.8 Effect of different concentrations of WAF exposure on the level of Hydroperoxide in different tissues of *O.mossambicus***

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 27.52 ± 3.07 | 14.85 ± 1.92 | 13.74 ± 2.16 | 5.71 ± 1.16 |
| 1.5ppm | 30.76 ± 3.77 | 16.24 ± 2.04 | 17.21 ±2.19 | 6.93 ± 1.09 |
| 3.0ppm | 38.55 ± 4.91 | 22.73 ± 2.79 | 25.57 ± 4.26 | 9.85 ± 1.88 |
| 5.0ppm | 43.93 ± 5.18 | 31.25 ± 3.11 | 34.48 ± 5.12 | 14.76 ± 2.15 |

⇒ Values are expressed as m moles / 100 g wet tissue.

⇒ Average of six values in each groups ± SD.

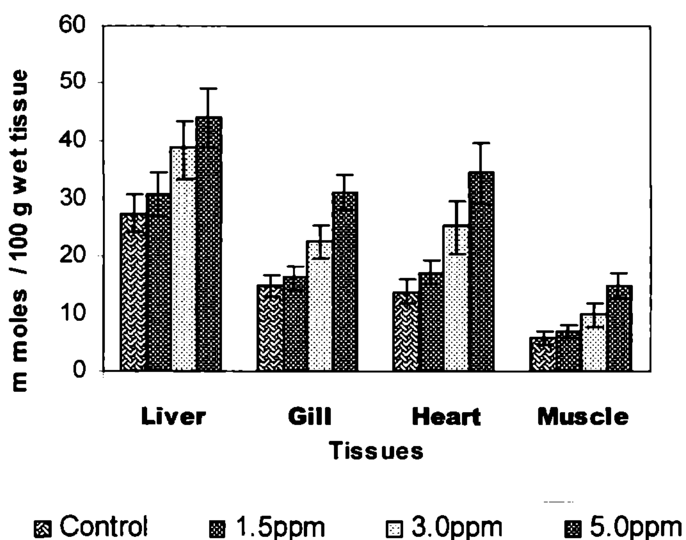



**Figure 4.10 Levels of hydroperoxide in the various tissues exposed to different concentrations of WAF**

**Table 4.8 a Two-Factor ANOVA for Hydroperoxide**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 592.6692 | 3 | 197.5564 | 37.23845 | 2.11E-05 | 3.862539 |
| Between tissues | 1343.921 | 3 | 447.9736 | 84.44092 | 6.55E-07 | 3.862539 |
| Error | 47.74655 | 9 | 5.305172 | | | |
| Total | 1984.337 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 4.8 a) and LSD analysis (Tables 4.9 b) revealed that a significant increase ($P<0.001$) in hydroperoxide level in all the WAF treated groups, except 1.5ppm, when compared to the control group. LSD value at 5% level was 3.684. Further comparison using LSD analysis showed that there was a significant change among different concentration treated groups. Comparison between tissues (Table 4.9 c) also showed a significant change with the exception between heart and gill.

**Table 4.9 Effect of different concentrations of WAF exposure on the level of malondialdehyde in different tissues of *O. mossambicus*.**

| GROUPS | LIVER | GILL | HEART | MUSCLE |
|---|---|---|---|---|
| Control | 0.143 ± 0.038 | 0.094 ± 0.028 | 0.112 ±0.036 | 0.054 ± 0.018 |
| 1.5ppm | 0.138 ± 0.044 | 0.088 ± 0.033 | 0.109 ± 0.037 | 0.050 ± 0.017 |
| 3.0ppm | 0.151 ± 0.057 | 0.102 ± 0.045 | 0.121 ± 0.031 | 0.049 ± 0.019 |
| 5.0ppm | 0.445 ± 0.050 | 0.276 ± 0.040 | 0.334 ± 0.038 | 0.098 ± 0.030 |

⇒ Values are expressed as m mol / 100 g wet tissue.

⇒ Average of six values in each groups ± SD.

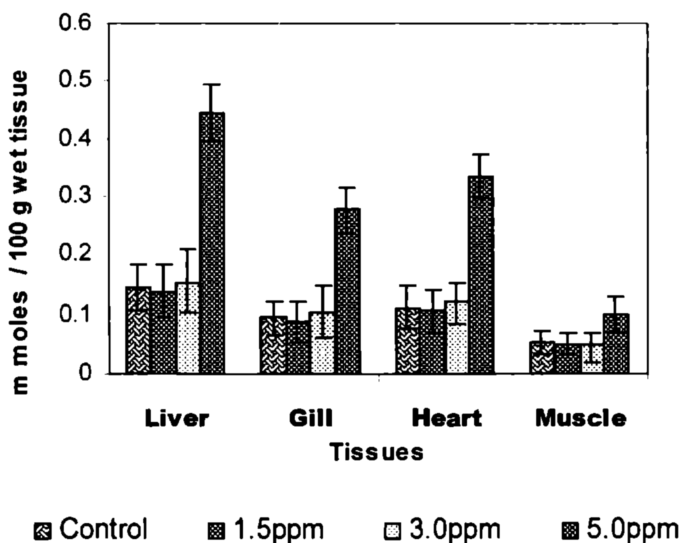



**Figure 4.11 Levels of malondialdehyde in the various tissues exposed to different concentrations of WAF**

**Table 4.9 a Two-Factor ANOVA for malondialdehyde**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 0.105462 | 3 | 0.035154 | 12.46424 | 0.001480 | 3.862539 |
| Between tissues | 0.051396 | 3 | 0.017132 | 6.074281 | 0.015168 | 3.862539 |
| Error | 0.025383 | 9 | 0.00282 | | | |
| Total | 0.182241 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares.

Two-Factor ANOVA (Table 4.9 a) and further LSD analysis (Table 4.9 b) revealed that there was a significant increase (P<0.001) in malondialdehyde level only between control and 5.0ppm WAF treated group. Whereas 1.5ppm and 3.0ppm treated group did not show any significant difference .when compared to control. LSD value at 5% level was 0.0849. Comparisons between the concentrations showed significant increase, except between 3.0ppm and 1.5ppm WAF treated groups. LSD analysis (Table 4.9 c) also revealed that there was no significant difference between tissues with the exception of liver and muscle, as well as muscle and heart.

**Table 4.9 b Results of LSD analysis for different WAF concentrations**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | GSH | CD | HP | MDA |
| Control Vs 1.5ppm | P < 0.05 | NS | NS | NS |
| Control Vs 3.0ppm | P < 0.001 | P < 0.05 | P < 0.001 | NS |
| Control Vs 5.0ppm | NS | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 1.5ppm | NS | P < 0.001 | P < 0.001 | NS |
| 5.0ppm Vs 1.5ppm | P < 0.01 | P < 0.001 | P < 0.001 | P < 0.001 |
| 5.0ppm Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |

**Table 4.9 c Results of LSD analysis for different tissues**

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | GSH | CD | HP | MDA |
| Liver Vs Gill | NS | P < 0.001 | P < 0.001 | NS |
| Liver Vs Heart | P < 0.001 | P < 0.001 | P < 0.001 | NS |
| Liver Vs Muscle | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| Heart Vs Gill | P < 0.001 | P < 0.05 | NS | NS |
| Muscle Vs Gill | P < 0.001 | P < 0.05 | P < 0.001 | NS |
| Muscle Vs Heart | P < 0.05 | P < 0.001 | P < 0.001 | P < 0.05 |

NS Not significant
CD Conjugated dienes
MDA Malondialdehyde
HP Hydroperoxide
GSH Glutathione

## 4D. DISCUSSION

Petroleum hydrocarbons are important pollutants in aquatic ecosystems. They are readily taken up from the water column, sediments and food sources into the liver and other tissues of fish (Walker and Livingstone, 1992). During polycyclic aromatic hydrocarbon metabolism, quinones and other redox cycling derivatives may be formed which are chemically unstable and have the potential to produce reactive oxygen species by redox cycling and can cause oxidative stress (Lemaire and Livingstone, 1997; Lemaire *et al.*, 1994; Washburn and Di Giulio, 1989). Reducing equivalents for this cycles are readily available from the flavoprotein NAD(P)H- dependent cytochrome $P_{450}$ reductase, NADH being the preferred substrate (Lemaire and Livingstone 1994, 1997).

As reported by Kappus (1986) and Di Giulio *et al.* (1989), antioxidant defenses consist of enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) and water soluble reductants such as glutathione. One of the important features of these enzymes is their inducibility under conditions of oxidative stress, and such induction can be important adaptation to petroleum hydrocarbon-induced stress (Akcha *et al.*, 2000). The results obtained in the present study reveal that SOD, CAT and GPX appeared to be significantly elevated in *O.mossambicus* exposed to 1.5ppm and 3.0ppm concentrations of water-accommodated fractions of crude oil for 21 days. SOD, CAT and GPX are considered as the primary antioxidant enzymes which help in the detoxification of reactive oxygen

species formed from petroleum hydrocarbons by decreasing the peroxide levels or by maintaining a steady supply of metabolic intermediate like glutathione (GSH) and NADHP (Kappus, 1985). The tissue specific increase in SOD, CAT and GPX activities shows the following trend: Gill > Liver > Heart > Muscle. The increased activities of SOD, CAT and GPX in gills may be related to their physiological role in respiration. In fact, in fish the extraction of oxygen from water occurs primarily at the gill surface and thereby the gills posses a more rapid and efficient enzymatic mechanism against increased levels of oxygen radicals (Afonso *et al.*, 1996). The increase in tissue SOD activity suggests an increased generation of intracellular hydrogen peroxide that could be adequately detoxified by CAT and GPX activities which was also significantly higher in gill, liver, heart and muscle tissues of *O.mossambicus* when exposed to 1.5ppm and 3.0ppm of water-accommodated fractions of crude oil for 3 weeks. Reduction of superoxide anion radicals by SOD and of hydrogen peroxide and organic hydroperoxides by CAT and GPX respectively prevent the formation of free radical intermediates from petroleum hydrocarbons by oxygen reduction mechanisms (Reddy, 1997). Hong *et al.* (2002); Livingstone, (2001) and Rudolph *et al.* (2002) have also independently reported similar increase in antioxidant enzymes in *Pagrosomus major L. limanda, Z ophiocephalus* and also in juvenile trout, *Oncorhynchus mykiss* exposed to petroleum hydrocarbons. Thus the increased activities of SOD, CAT and GPX in the present study are known to serve as protective responses to eliminate reactive free radicals formed from petroleum hydrocarbons and thereby

protect the cell from further injury (Karakoc *et al.*, 1997; Sachins *et al.*, 1997; Sole *et al.*, 1998).

The results of this study also demonstrated that the activities of glutathione reductase (GR) and glutathione-S-transferase (GST) were increased when compared to control groups, in a statistically significant fashion in *O.mossambicus* when exposed to 1.5ppm and 3.0ppm of water-accommodated fractions of crude oil for 3 weeks. The maximum increase was seen in the liver (liver plays a central role in petroleum hydrocarbons biotransformation processes (Buhler and Williams, 1988) followed by gill, heart and muscle. The increased activity of GR in 1.5ppm and 3.0ppm WAF dosed experimental animal shows that GSSG production is high and GPX activity is also high. The increased activity of GST-a multifunctional protein suggests that it increases the conjugation of epoxides of polycyclic aromatic hydrocarbons with the tripeptide glutathione, the resulting conjugates being water soluble and thus more easily excretable, thereby minimizing the cellular damages the petroleum hydrocarbons cause (Camus *et al.*, 2002). In addition, the increased GST activity serves to reduce the likelihood of electrophilic compounds (epoxide of polycyclic aromatic hydrocarbons) covalently binding to important cellular macro molecules such as DNA (Cheung *et al.*, 2001). Gadagbui *et al.* (1996) also support the view that *O.mossambicus* is more likely to excrete xenobiotics as glutathione conjugates or mercapturic acids because of its high GST activity.

GSH, as an oxyradical scavenger, is important in antioxidant defense. GSH is a co-substrate for hydrogen peroxide and hydroperoxide

decomposition to their corresponding alcohols, through GPX activity (Sies, 1993) and plays an important role in the biotransformation of polycyclic aromatic hydrocarbons. GSH acts as a reactant in conjugation with epoxide of polycyclic aromatic hydrocarbons, catalyzed by GST, for subsequent excretion (Gallagher and Di Giulio, 1992). In the present study also a significant increase in GSH was observed in liver, gill, heart and muscle tissues of *O.mossambicus* exposed to 1.5ppm and 3.0ppm of water-accommodated fractions of crude oil. Increase in GSH levels is a very important indicator of the detoxification ability of WAF dosed *O.mossambicus*. Thus it is conceivable that higher activities of GSH, GR and GST in 1.5ppm and 3.0ppm WAF dosed experimental animals may represent a first line of defense against tissue damage.

Lipid peroxidation, as measured by the concentration of malondialdehyde, was not significantly increased in tissues of fish exposed to 1.5ppm and 3.0ppm of WAF when compared to the control group. This could be due to the significant increase of non-enzymatic antioxidant namely glutathione as well as by the increased activity of GST, which can prevent the formation of malondialdehyde (Christophersen, 1986).

On the other hand, activities of SOD, CAT, GPX, GR and GST as well as GSH were significantly inhibited in fish exposed to 5.0ppm of WAF for 21 days. The decreased SOD and CAT activity observed in 5.0ppm WAF dosed experimental animals may be related to the increase in $O_2^-$ production. CAT inhibition by $O_2^-$ was previously described by Kono and Fridovich (1982). This shows that in 5.0ppm WAF dosed experimental animals, tissue SOD, CAT and GPX adaptive response would not be enough

to protect cells against the damage by oxyradicals generated from petroleum hydrocarbons, whereas the levels of conjugated dienes, hydroperoxides and malondialdehyde were significantly increased in fish exposed to 5.0ppm of WAF when compared to control group. The maximum increase was observed in the liver followed by gill, heart and muscle tissues. Albaiges *et al.* (2000) have also reported similar increase in malondialdehyde activity in mussels (*Mytilus edulis*) collected near the wreck point six months after the spillage of Aegean Sea Oil. The significant increase in malondialdehyde in 5.0ppm WAF dosed fish in this study might be due to the enhanced generation of reactive oxygen species and also due to the decreased levels of GSH and GST (Francesco *et al.,* 1998).

The foregoing result shows that lower doses (1.5ppm and 3.0ppm) of water-accommodated fractions of crude oil-induced oxidative stress in *O.mossambicus* which might be overcome to a large extent by an increased production of the chain-breaking antioxidant-GSH as well as with its antioxidant defense mechanism. On the contrary, in 5.0ppm WAF dosed fishes coping mechanism has not set in even at the end of 21 days, and detoxication and the removal of electrophile has not been effected.

The aforesaid evidences project the fact that exposure of *O.mossambicus* to petroleum hydrocarbons for three weeks in stressful and oxidative stress-related parameters can be adapted for future investigations, as biomarkers of petroleum hydrocarbons damage to aquatic organisms.

# *Chapter* 5

# Effects Of Petroleum Hydrocarbons On Lysosomal Membrane And Haematological Parameters

## 5.1 STUDIES ON LYSOSOMAL MEMBRANE STABILITY

### 5.1A. INTRODUCTION

Lysosomes are subcellular organelles which are bounded by a semi permeable lipoprotein membrane, containing a battery of over sixty hydrolases such as acid phosphatase (ACP) and β-glucuronidase, acting optimally at an acid pH, which is maintained by a membrane $Mg^{2+}$ ATPase dependent $H^{+}$ ion proton pump (Okhuma *et al.,* 1982). Lysosomes are collectively capable of degrading all classes of macromolecules of intracellular and extra cellular origin (Lowe and Fossovu, 2000).

Lysosomal damage is well established as a biomarker of petroleum hydrocarbons stress for a diverse range of marine vertebrate and invertebrate animals (Moore, 1990; Tabata *et al.*, 1990). Lysosomes accumulate wide range of chemical contaminants including polynuclear aromatic hydrocarbons which can lead to membrane damage resulting in the leakage of their resident acid hydrolases into the cytosol and damage the cells (Lowe, 1981). The effect of chemicals on a lysosome membrane thus can be evaluated by measuring the activity of released enzymes (Dean, 1981). Exposure of marine mollusks to petroleum hydrocarbons has been demonstrated to result in the destabilization of lysosomal membrane, if the storage capacity is overloaded, with subsequent increase in the activities of certain lysosomal enzymes, notably, acid phosphatase and β-glucuronidase (Moore, 1985; Pipe, 1986). Injection of polynuclear aromatic hydrocarbons (Bayne *et al.,* 1979) as well as exposure to water-soluble fractions of crude oil from the Auk field (Moore, 1980) experiments in the digestive cells of *Mytilus* showed that lysosomal stability

was reduced. This indicates that the stressors could induce functional alterations in the lysosomes and in certain instances structural changes, which were indicative of cytotoxicity (Moore *et al.*, 1978).

The sensitivity of lysosomes to environmental pollutants including petroleum hydrocarbons suggests that lysosomal responses may be considered as early warning systems for detection of the disturbances in the surroundings. The lysosomal stability measured in terms of the lysosomal enzyme release assay, thus can clearly reflect any breakdown in the adaptive capacity of the fish to WAF of crude oil injury. Thus, a test battery measuring lysosomal perturbations may be recommended as a tool for biological effect monitoring (Kohler, 1991).

In this chapter, effort has been made to extend the lysosomal enzyme release assay technique to *Oreochromis mossambicus* and to assess its effectiveness as a sensitive index of petroleum hydrocarbons.

## 5.1B. MATERIALS AND METHODS

Collection and acclimation of fish, method of WAF preparation and dosing and collection of liver tissue was the same as that described in Chapter 2 B. The release of typical lysosomal enzymes-β-glucuronidase and acid phosphatase from the lysosomes rich fraction of the liver has been taken as a measure of lysosomal stability. The liver tissues of the control and WAF dosed fishes for three weeks under experimental conditions were used for the lysosomal stability experiment, after the liver tissue was washed in ice-cold 0.33 M isotonic sucrose, blotted dry and weighed.

### 5.1B.1 Assay of β-Glucuronidase (E.C.3.2.1.31)

**a. Activity of β-glucuronidase in the various subcellular fractions of liver tissue.**

Liver was homogenized in 0.33 M sucrose at 0°C (10%) and the homogenate was centrifuged at 600 g for 10 min in a high speed refrigerated centrifuge. The sediment of nuclei, unbroken cells and plasma membrane (nuclear fraction) was separated. The supernatant was again centrifuged at 15000 g for 30 min. The 15000 g sediment (lysosomal rich fraction) and nuclear fraction were resuspended in citrate buffer containing 0.2% Brij-35. The 15000 g supernatant (soluble fraction) was diluted with an equal volume of double strength buffer. The activity of β-glucuronidase and acid phosphatase- typical lysosomal enzymes were determined in all these fractions (Plummer, 1987).

**b. Rate of release of β-Glucuronidase from the lysosomal-rich fraction of liver or Lysosomal Enzyme Release Assay.**

***(i) In vitro* Studies**

Liver from control and different concentration of WAF dosed fishes were homogenized in cold isotonic sucrose and the lysosomal fraction was obtained as above. The lysosomal pellet was washed, centrifuged at 15000 g 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 min. The retrieved fractions were stored immediately at 0°C (control). In order to study the effect of WAF on the lysosomal membrane, a definite volume of the lysosomal

suspension (Test) was incubated in presence of each concentration of WAF such that the final WAF concentrations in the tubes were 1.5ppm, 3.0ppm and 5.0ppm respectively in the mixture. Here also aliquots were withdrawn at time intervals of 0, 15, 30 and 45 min. Both the control and test aliquots were centrifuged at 15000 g for 30 min to separate the unbroken lysosomes and β-glucuronidase activity released into the supernatant was determined. Total activity in the lysosomes-rich fraction was estimated after adding citrate buffer containing Brij-35 after appropriate dilution.

***(ii) In vivo* Studies**

Hepatic tissue from control fish and from those exposed to WAF for three weeks were homogenized separately in isotonic sucrose and centrifuged to obtain the lysosomal fraction as described in 5.1B.1a. The rate of release of β-glucuronidase was noted at definite time intervals by withdrawing definite aliquots of the suspension as described in *in vitro* methods in 5.1B.1b (i).

**c. Determination of β-Glucuronidase activity (Kawai and Anno, 1971)**

p-nitrophenyl-β-D-glucuronide in 0.1 M citrate buffer (pH 4.5) was used as the substrate. The reaction system containing substrate and enzyme source was incubated at room temperature for 30 min and the reaction was stopped by adding 0.2 N sodium carbonate solution. The absorbance was read at 400 nm in a UV-visible spectrophotometer. The enzyme activity is expressed in terms of μg of p-nitrophenol liberated per hour per gram protein using p-nitrophenol as standard. Protein was estimated by the method of Lowry *et al.* (1951).

### 5.1B.2 Assay of Acid Phosphatase (ACP)

**a. Activity of Acid Phosphatase in the various subcellular fractions of liver tissue.**

Activity of acid phosphatase in the various subcellular fractions of liver tissue was determined by the procedure described in 5.1B.1a.

**b. Rate of release of Acid Phosphatase from the lysosomal fraction of liver or Lysosomal Enzyme Release Assay**

***(i) In vitro* Studies**

***(ii) In vivo* Studies**

The *in vitro* and *in vivo* effects of WAF exposure on the rate of release of acid phosphatase from hepatic lysosomes of normal and WAF treated experimental animals were studied by the procedure described in the 5.1B.1b (i) (ii).

**c. Determination of Acid Phosphatase (ACP) activity (Anon, 1963)**

0.5 ml of p-nitro phenyl phosphate (400 mg %) was mixed with an equal volume of 0.1 M citrate buffer of pH 4.8. The enzyme was added and incubated for 30 min at room temperature. At the end of 30 min, reaction was stopped by the addition of 4 ml of 0.1 N NaOH. The absorbance of the solution was measured at 410 nm in a UV-visible spectrophotometer (Hitachi). The amount of p-nitrophenol liberated by the acid phosphatase per hour per mg protein gives the specific activity. Protein was estimated by the method of Lowry et al. (1951).

**d. Statistical Analysis**

Statistical analysis of results for lysosomal stability was done by ANOVA and subsequent comparisons by Least Significant Difference (LSD) (Zar, 1996)

## 5.1C. RESULTS

### (a) Subcellular activity of β-glucuronidase

The subcellular activity of β-glucuronidase is given in Table 5.1.1 and Figure 5.1.1.

**Table 5.1.1 Effect of different concentrations of WAF exposure on the subcellular activity of β-glucuronidase *in vivo* in *O.mossambicus***

| GROUPS | β-glucuronidase activity in Hepatic Tissue | | | |
|---|---|---|---|---|
| | Nuclear Fraction | Lysosomal Fraction | Soluble Fraction | Lysosomal to Soluble Activity |
| Control | 4.55 ± 0.74 | 33.25 ± 2.12 | 14.85 ± 1.84 | 2.239 ± 0.15 |
| 1.5ppm | 6.03 ± 0.29 | 29.48 ± 1.08 | 16.46 ± 1.22 | 1.791 ± 0.10 |
| 3.0ppm | 7.25 ± 0.39 | 24.79 ± 0.58 | 20.73 ± 2.34 | 1.195 ± 0.08 |
| 5.0ppm | 8.05 ± 0.22 | 17.25 ± 0.67 | 26.65 ± 2.21 | 0.647 ± 0.04 |

⇒ Activities are expressed as mg p-nitrophenol liberated / h / g protein in each fraction.
⇒ Average of six values in each groups ± SD.

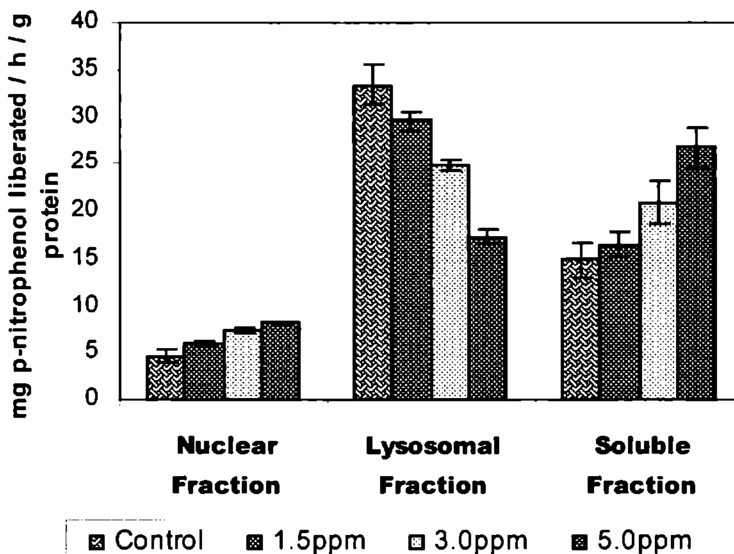



**Figure 5.1.1 Subcellular activity of β-glucuronidase in *O.mossambicus***

One way ANOVA showed that there was an overall significant change ($P<0.001$) in the β-glucuronidase activity in the nuclear (F-66.82) (Table 5.1.1a), soluble (F=29.19) (Table 5.1.1 b) and lysosomal (F=176.92) (Table 5.1.1c) fractions of the different concentrations of WAF dosed experimental animals when compared to control.

**Table 5.1.1 a ANOVA for nuclear β-glucuronidase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 41.9088 | 3 | 13.9696 | 66.82 | 1.34E-10 | 3.098393 |
| Within concentrations | 4.1812 | 20 | 0.20906 | | | |
| Total | 46.09 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.1.1 b ANOVA for soluble β-glucuronidase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 499.0028 | 3 | 166.3343 | 29.19 | 1.67E-07 | 3.098393 |
| Within concentrations | 113.9841 | 20 | 5.699207 | | | |
| Total | 612.987 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.1.1 c ANOVA for lysosomal β-glucuronidase activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 855.5329 | 3 | 285.1776 | 176.92 | 1.45E-14 | 3.098393 |
| Within concentrations | 32.23853 | 20 | 1.611927 | | | |
| Total | 887.7714 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

A subsequent comparison between different concentrations of WAF was done by Least Significance Difference (LSD) and the results of which are given in Table 5.1.1d.

**Table 5.1.1d Results of LSD analysis for subcellular β-glucuronidase activity**

| GROUPS | P Value | | |
|---|---|---|---|
| | NUCLEAR | LYSOSOMAL | SOLUBLE |
| Control x 1.5ppm | P < 0.001 | P < 0.001 | NS |
| Control x 3.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| Control x 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5ppm x 3.0ppm | P < 0.001 | P < 0.001 | P < 0.05 |
| 3.0ppm x 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5ppm x 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |

NS-Not Significant.

Studies on the effect of various concentrations of WAF exposure on β-glucuronidase activity in different subcellular fractions of liver showed a significant decrease (P<0.001) in lysosomal β-glucuronidase activity when compared to control. Whereas there was a significant increase (P<0.001) in nuclear β-glucuronidase activity when compared to control. However there was no significant increase in soluble enzyme activity when 1.5ppm treated groups were compared to control. But 3.0ppm and 5.0ppm WAF treated groups when compared to control revealed a significant (P<0.001) difference. LSD values of the nuclear, soluble and lysosomal β-glucuronidase fractions were 0.55, 2.88 and 1.53 respectively. Comparison between different WAF concentrations with respect to different fractions also revealed a significant change (P<0.001) at 5% level. Due to different concentrations of WAF exposure to the experimental animals resulted in decrease in β-glucuronidase activity when compared to control but there was a steady increase in soluble fraction activity. The ratio of β-glucuronidase activity in lysosomal fraction to that in the soluble fraction or lysosomal stability index

(LSI) was the lowest for 5.0ppm treated group (0.647), followed by 3.0ppm treated group which had an LSI of 1.195 and minimum for 1.5ppm WAF treated group which had an LSI of 1.791 whereas for control LSI was 2.239. The results indicate that when the concentrations of WAF increase, it causes greater damage or destabilization of the integrity of the lysosomal membrane which results in the activation and release of β-glucuronidase which is a lysosomal degradative enzyme.

**b. Lysosomal enzyme release assay *(in vitro and in vivo)***

The time dependent release of β-glucuronidase *in vitro* and *in vivo* is given in Tables 5.1.2 and 5.1.3.

**Table 5.1.2 Lysosomal enzyme (β-glucuronidase) release assay *in vitro***

| TIME (min) | CONTROL | 1.5ppm | 3.0ppm | 5.0ppm |
|---|---|---|---|---|
| 0 | 1.84 ± 0.082 (14.76) | 2.16 ± 0.05 (17.27) | 2.74 ± 0.21 (21.90) | 3.04 ± 0.03 (24.35) |
| 15 | 2.14 ± 0.11 (17.17) | 2.83 ± 0.22 (22.68) | 3.00 ± 0.32 (24.03) | 3.66 ± 0.07 (29.28) |
| 30 | 2.88 ± 0.32 (23.07) | 3.42 ± 0.08 (27.34) | 3.85 ± 0.32 (30.85) | 4.22 ± 0.09 (33.81) |
| 45 | 3.01 ± 0.08 (24.11) | 3.82 ± 0.19 (30.60) | 4.11 ± 0.15 (32.91) | 4.63 ± 0.08 (37.00) |

In brackets is represented β-glucuronidase release as % of total activity.

⇒ The values are expressed as mg / p-nitrophenol / h / g protein.

⇒ Average of six values in each groups ± SD.

**Table 5.1.2 a Two-Factor ANOVA for Lysosomal enzyme release assay *in vitro***

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between time | 5.150252 | 3 | 1.71675 | 139.06 | 7.41E-08 | 3.862539 |
| Between concentrations | 4.303322 | 3 | 1.43444 | 116.19 | 1.63E-07 | 3.862539 |
| Error | 0.111112 | 9 | 0.01234 | | | |
| Total | 9.564686 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

**Table 5.1.3 Lysosomal enzyme release assay (β-glucuronidase) *in vivo***

| TIME (min) | CONTROL | 1.5ppm | 3.0ppm | 5.0ppm |
|---|---|---|---|---|
| 0 | 0.98 ± 0.03 (7.88) | 1.36 ± 0.13 (10.85) | 1.93 ± 0.13 (15.43) | 2.01 ± 0.12 (16.17) |
| 15 | 1.55 ± 0.13 (12.39) | 1.60 ± 0.11 (12.83) | 2.15 ± 0.16 (17.25) | 2.64 ± 0.13 (21.10) |
| 30 | 2.43 ± 0.15 (19.49) | 2.88 ± 0.27 (23.02) | 3.37 ± 0.22 (27.00) | 3.85 ± 0.18 (30.78) |
| 45 | 2.67 ± 0.03 (21.36) | 3.01 ± 0.40 (24.11) | 3.75 ± 0.11 (30.09) | 4.12 ± 0.08 (32.99) |

In brackets is represented β-glucuronidase release as % of total activity.

⇒ The values are expressed as mg / p-nitrophenol / h / g protein.

⇒ Average of six values in each groups ± SD.

**Table 5.1.3 a Two-Factor ANOVA for Lysosomal enzyme release assay *in vivo***

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between time | 9.277857 | 3 | 3.092619 | 183.00 | 2.21E-08 | 3.862539 |
| Between concentrations | 3.805772 | 3 | 1.268591 | 75.07 | 1.09E-06 | 3.862539 |
| Error | 0.152088 | 9 | 0.016899 | | | |
| Total | 13.23572 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

Two-Factor ANOVA revealed that there was an overall significant change (P<0.001) between time intervals and also between different WAF concentrations (Table 5.1.3 a). Subsequent comparison by LSD analysis showed that there was a significant change (P<0.001) between different concentrations with the control and also among themselves. LSD value at 5% level was 0.1778 (*in vitro*) and 0.2079 (*in vivo*). Similarly there was a significant increase (P<0.001) in the rate of release of β-glucuronidase enzyme with different time intervals when compared with zero time and also among themselves. As the time interval increases both *in vitro* and *in vivo* studies showed that the rate of release of β-glucuronidase also significantly

increases. LSD analysis for lysosomal β-glucuronidase release *in vivo* and *in vitro* studies is given in Table 5.1.3 b.

**Table 5.1.3 b Results of LSD analysis for *in vitro* and *in vivo* studies**

| β-glucuronidase release with time (in minutes) | P value |
|---|---|
| 0-15 | $P < 0.05$ |
| 0-30 | $P < 0.001$ |
| 0-45 | $P < 0.001$ |
| 30-15 | $P < 0.05$ |
| 45-15 | $P < 0.05$ |
| 45-30 | $P < 0.05$ |

**(b) Subcellular activity of Acid phosphatase (ACP)**

The subcellular activity of acid phosphatase in hepatic tissue is given in Table 5.1.4 and Figure 5.1.2.

**Table 5.1.4 Effect of different concentrations of WAF exposure on the subcellular activity of ACP *in vivo* in *O. mossambicus***

| GROUPS | ACP activity in hepatic tissue | | | |
|---|---|---|---|---|
| | Nuclear Fraction | Lysosomal Fraction | Soluble Fraction | Ratio of Lysosomal to Soluble Activity |
| Control | 8.57 ± 0.44 | 16.98 ± 0.38 | 8.77 ± 0.25 | 1.928 ± 0.06 |
| 1.5ppm | 10.76 ± 0.80 | 12.95 ± 0.14 | 12.68 ± 0.39 | 1.012 ± 0.05 |
| 3.0ppm | 12.84 ± 0.62 | 8.45 ± 0.45 | 18.15 ± 0.46 | 0.0469 ± 0.06 |
| 5.0ppm | 17 18 ± 0.41 | 4.99 ± 0.24 | 20.91 ± 0.64 | 0.248 ± 0.04 |

⇒ Activities are expressed as mg p-nitrophenol liberated / h / g protein in each fraction.

⇒ Average of six values in each groups ± SD.

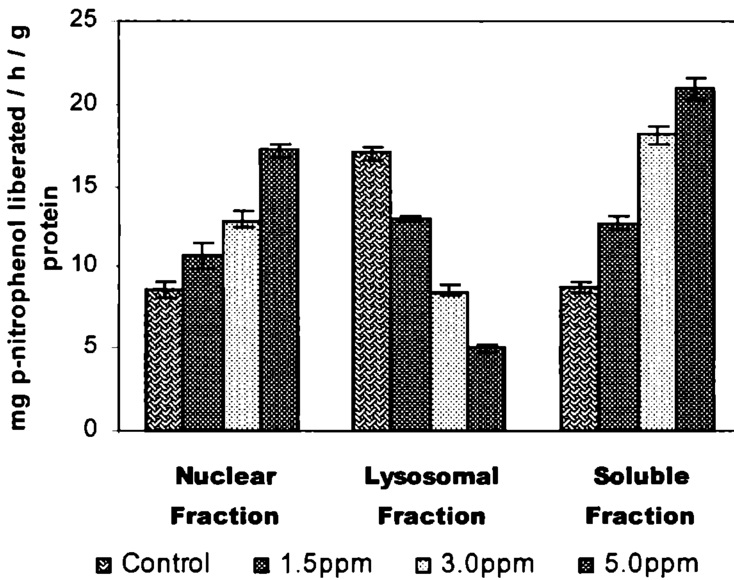



**Figure 5.1.2 Subcellular activity of acid phosphatase in *O.mossambicus.***

**Table 5.1.4 a ANOVA for nuclear ACP activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Concentrations | 242.3846 | 3 | 80.79487 | 233.4881 | 9.93E-16 | 3.098393 |
| Within Concentrations | 6.920683 | 20 | 0.346034 | | | |
| Total | 249.3053 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.1.4 b ANOVA for soluble ACP activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Concentrations | 534.1794 | 3 | 178.0598 | 660.9741 | 6.63E-20 | 3.098393 |
| Within Concentrations | 5.3878 | 20 | 0.26939 | | | |
| Total | 539.5672 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.1.4 c ANOVA for lysosomal ACP activity**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Concentrations | 492.5926 | 3 | 164.1975 | 1551.253 | 7.6E-24 | 3.098393 |
| Within Concentrations | 2.116967 | 20 | 0.105848 | | | |
| Total | 494.7095 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

One way ANOVA showed that there was an overall significant change (P<0.001) in the ACP activity in the nuclear (F=233.488) (Table 5.1.4 a), soluble (F=660.974) (Table 5.1.4 b) and lysosomal (F=1551.253) (Table 5.1.4 c) fractions of the experimental animals. Subsequent comparisons between different groups by LSD analysis revealed that (Table 5.1.4 d) there was a significant increase (P<0.001) in nuclear and soluble ACP in all WAF treated groups when compared to the control. Whereas a significant decrease (P<0.001) was observed in lysosomal ACP in all WAF treated groups when compared to control.

**Table 5.1.4d Results of LSD analysis for Subcellular acid phosphatase activity**

| GROUPS | P value | | |
|---|---|---|---|
| | NUCLEAR | LYSOSOMAL | SOLUBLE |
| Control x 1.5ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| Control x 3.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| Control x 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5ppm x 3.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 3.0ppm x 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5ppm x 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 |

## b. Lysosomal enzyme release assay (*in vitro and in vivo)*

The time dependent release of ACP *in vitro and in vivo* is given in Tables 5.1.5 and 5.1.6.

**Table 5.1.5 Lysosomal enzyme (Acid Phosphatase) release assay *in vitro***

| TIME (min) | CONTROL | 1.5ppm | 3.0ppm | 5.0ppm |
|---|---|---|---|---|
| 0 | 0.78 ± 0.03 (7.65) | 0.93 ± 0.02 (9.12) | 1.19 ± 0.07 (11.67) | 1.23 ± 0.07 (12.06) |
| 15 | 1.16 ± 0.11 (11.37) | 1.18 ± 0.12 (11.57) | 1.42 ± 0.13 (13.92) | 1.85 ± 0.12 (18.14) |
| 30 | 1.24 ± 0.15 (12.16) | 1.92 ± 0.13 (13.92) | 1.96 ± 0.03 (19.22) | 2.11 ± 0.06 (20.69) |
| 45 | 1.31 ± 0.07 (12.84) | 1.98 ± 0.06 (21.18) | 2.16 ± 0.04 (21.23) | 2.35 ± 0.06 (23.04) |

In brackets is represented ACP release as % of total activity.

⇒ The values are expressed as mg / p-nitrophenol / h / g protein.

⇒ Average of six values in each groups ± SD.

**Table 5.1.5 a Two-Factor ANOVA for Lysosomal enzyme release assay *in vitro***

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between time | 2.063419 | 3 | 0.687806 | 24.57 | 0.000114 | 3.862539 |
| Between concentrations | 1.259119 | 3 | 0.419706 | 14.99 | 0.000758 | 3.862539 |
| Error | 0.251906 | 9 | 0.02799 | | | |
| Total | 3.574444 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

*In vitro* studies showed an overall significant change ($P<0.001$) between ACP release with time and also between different concentrations by ANOVA (Table 5.1.5 a). Further analysis by LSD revealed (Table 5.1.6 b) a significant difference in the release of ACP at time intervals of 0-15, 0-30, 0-45 and 15-30 min. However there was no significant change between 30-45

min. LSD values at 5% level was 0.2676. Subsequent comparisons (Table 5.1.6 b) showed that the rate of release of ACP was significantly different (P<0.001) in all the WAF exposed animals when compared with the control. However there was no significant difference between 3.0ppm and 1.5ppm as well as 5.0ppm and 3.0ppm treated groups. The maximum ACP release was by 5.0ppm treated groups.

**Table 5.1.6 Lysosomal enzyme (ACP) release assay *in vivo***

| TIME (min) | CONTROL | 1.5ppm | 3.0ppm | 5.0ppm |
|---|---|---|---|---|
| 0 | 0.63 ± 0.048 (6.19) | 0.75 ± 0.02 (7.36) | 0.99 ± 0.01 (9.76) | 1.03 ± 0.02 (10.11) |
| 15 | 1.01 ± 0.008 (9.92) | 1.04 ± 0.004 (10.01) | 1.15 ± 0.005 (11.23 | 1.21 ± 0.02 (11.90) |
| 30 | 1.18 ± 0.19 (12.10) | 1.36 ± 0.25 (13.35) | 1.42 ± 0.05 (13.95) | 1.65 ± 0.06 (16.21) |
| 45 | 1.33 ± 0.11 (12.95) | 1.40 ± 0.07 (13.71) | 1.44 ± 0.03 (14.09) | 1.72 ± 0.01 (16.90) |

In brackets is represented ACP release as % of total activity.

⇒ The values are expressed as mg / p-nitrophenol / h / g protein.

⇒ Average of six values in each groups ± SD.

**Table 5.1.6 a Two-Factor ANOVA for Lysosomal enzyme (ACP) release assay *in vivo***

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between time | 1.000084 | 3 | 0.333361 | 75.77 | 1.05E-06 | 3.862539 |
| Between concentrations | 0.288688 | 3 | 0.096229 | 21.87 | 0.000181 | 3.862539 |
| Error | 0.039599 | 9 | 0.0044 | | | |
| Total | 1.328371 | 15 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

*In vivo* experiments on lysosomal ACP release showed that there was an over all significant change (P<0.01) between time intervals and also between concentrations (Table 5.1.6 a). Subsequent LSD analysis revealed

(Table 5.1.6 b) that there was a significant increase ($P<0.001$) when different time intervals were compared with zero time. However there was no significant change between 30 minute and 45 minute intervals. LSD value at 5% level was 0.1061. Further comparison showed that there was a significant change ($P<0.001$) between control and groups treated with 3.0ppm and with 5.0ppm. However there was no significant change between control and 1.5ppm WAF dosed experimental animals. There was a significant change ($P<0.001$) among concentrations at 5% level.

**Table 5.1.6 b Results of LSD analysis for *in vitro* and *in vivo* studies**

| ACP release with time (in minutes) | P value |
|---|---|
| 0-15 | P < 0.05 |
| 0-30 | P < 0.001 |
| 0-45 | P < 0.001 |
| 30-15 | P < 0.05 |
| 45-15 | P < 0.05 |
| 45-30 | NS |

NS- Not significant

## 5.1D. DISCUSSION

One of the first alterations due to crude oil exposure that can be detected at lysosomal level is the variation in the lysosomal stability (Moore *et al.*, 1987) and these changes indicate severe dysfunction of lysosomal system. These interfere with the intracellular digestion of food, the normal turn over of proteins and the regulation of fusion processes associated with the lysosomal vacuolar system (Livingstone *et al.*, 2000). Injury resulting in

destabilization of the lysosomal lamina bears a quantitative relationship to the magnitude of petroleum hydrocarbons stress response (Bayne *et al.*, 1982).

Lysosomes play a crucial role in the isolation and sequestration of polynuclear aromatic hydrocarbons of crude oil, and thus relieve biological machinery from their toxic effects (Tzartsidze *et al.*, 1984, Lowe *et al.*, 2000). But once the storage capacity is exceeded, there ensues a leakage of hydrolytic lysosomal enzymes-β-glucuronidase and acid phosphatase into the cytoplasm and nucleoplasm bringing about a derangement of cell function (Moore, 1988). Lysosomal membrane stability is thus a highly sensitive measure of the functional state of the cell and provides an ideal starting point for probing into the generalized cellular deterioration. Assessment of this type of injury has been confirmed as an extremely sensitive index of cellular condition by Pickwell and Steinert, 1984.

In the present study, lysosomal β-glucuronidase and acid phosphatase activity in WAF dosed fishes was lower than that of control but the soluble fraction activity was significantly increased in all selected concentrations. Results of lysosomal enzyme release assay *in vitro* as well as *in vivo* revealed significant increase in β-glucuronidase and acid phosphatase release with time. This observation showed direc tinteraction of WAF of crude oil with the membrane lipids and proteins. The works by Nelson (1985) indicate that polynuclear aromatic hydrocarbons have shown to penetrate phospho lipid membrane and alter their physico-chemical properties, including membrane fluidity and permeability. Reduced lysosomal stability has previously been reported to contribute to impaired

immunocompetence and to autophagic loss of body tissue (Farnley *et al.*, 2000). The same conclusion that has been accomplished by the works of Krishna Kumar *et al.* (1997) also showed that lysosomal membrane stability was significantly decreased when digestive tissue of *Mytilus edulis* was exposed to microcapsulated polycyclic aromatic hydrocarbons for 30 days.

Lysosomes are noted for their accumulation of polycyclic aromatic hydrocarbons of crude oil that are known to be involved in the generation of oxygen radicals (Varanasi *et al.*, 1985). Polycyclic aromatic hydrocarbons entering the cell and hence to the interior of lysosomes may attack the unsaturated fatty acids of the membrane as an absolute step towards the process of lipid peroxidation. Petroleum hydrocarbons intensify the injury of the lysosomal membrane directly as well as indirectly causing release of degradative enzyme (Cajaraville *et al.*, 1991). As lipid peroxidation proceeds in lysosomal membrane due to oxyradical production from polycyclic aromatic hydrocarbons of crude oil interaction, several of the products modify the physical characteristics of biological membranes. When free fatty acids get damages membrane conformation is lost and may lead to gaps in the membrane (Thomas *et al.*, 2002). Peroxidiation of biological membrane can also cause cross-links between proteins to form high molecular weight aggregates within the membrane, probably due to free radical processes. This can eventually lead to change in membrane properties and loss of its bound enzymes. In general, the overall effects of lipid peroxidiation are: decrease in membrane fluidity and increase in the 'leakiness' of the membrane to substances that do not normally cross it (Kohler, 1991).

Further evidence of lysosomal destabilization comes from several ultrastructural studies of the effects of polycyclic aromatic hydrocarbons on secondary lysosomes in digestive cells (Nott *et al.*, 1985; Pipe *et al.*, 1986). They have demonstrated the presence of corrugation of the bounding membrane with possible associated blebbing activity. Increased frequency of membrane breaks has also been described indicative of the greater fragility of lysosomes from cells exposed to crude oil. Apparent leakage of lysosomal β-glucuronidase and acid phosphatase has been demonstrated in the case of lysosomes from the digestive cells of crude oil-exposed *L.littorea* (Pipe *et al.*, 1986).

In the present study also areas of massive necrosis was shown by gill tissue of WAF dosed experimental animals when subjected to histopathological examination (Chapter 7). This can be identified as one of the reasons for the labilisation of the lysosomal membrane leading to the spill of lysosomal β-glucuronidase and acid phosphatase to soluble fraction. The consequence of such a release of lysosomal marker enzymes is believed to lead to enhanced cell damage and possibly cell death (Ringwood *et al.*, 1998).

Thus it can be summarized that WAF of Bombay High crude oil damages the lysosomal system involving the loss of integrity of the lysosomal membranes, thereby resulting in 'leaky' lysosomes which can thus release their hydrolytic marker enzymes - β-glucuronidase and ACP into the cytosol.

## 5.2. STUDIES ON HAEMATOLOGICAL PARAMETERS

### 5.2A. INTRODUCTION

Since blood forms a good clinical diagnostic tool for assessing the pathological and physiological conditions in fish, certain haematological parameters like erythrocyte membrane stability, haemoglobin content, RBC count, WBC count and haematocrit value of the *O.mossambicus* exposed to 1.5ppm, 3.0ppm and 5.0ppm concentrations of WAF of Bombay High crude oil for three weeks were determined.

Erythrocytes lack organelles and are essentially composed of a single membrane, the plasma membrane, surrounding a solution of haemoglobin (this protein forms about 95% of the intracellular protein of RBC) (Robert *et al.,* 1998). Since they are free from membranes and organelles any effect of a metabolite or other agent on osmotic haemolysis can be interpreted as an effect on the plasma membrane. The stability of the erythrocyte membrane is a simple and precise method for evaluating the toxicity of pollutants because the release of Hb from RBC can serve as a criterion for haemolytic effects. A change in heamoglobin level, erythrocyte count, leucocyte count and haematocrit value are very characteristic findings in fish after toxic exposure to different pollutants (Wlasow and Dabrowska, 1990).

### 5.2B. MATERIALS AND METHODS

#### a. Erythrocyte membrane stability

Erythrocyte membrane stability was determined by the method of Seiman and Weinstein (1966) with slight modifications to suit the working conditions.

*O. mossambicus* of size (15 ± 3 g) were used for the erythrocyte membrane stability study. A stock suspension of erythrocytes was prepared from fresh blood drawn from the cardinal vein in syringes containing citrate as the anti coagulant (Michael *et al.*, 1994) and then it was centrifuged at 4°C in a refrigerated centrifuge at 5000 rpm for 20 min. The erythrocytes were then washed with isotonic (0.85%) saline solution. Then different volumes of the suspension was mixed with distilled water to hemolyse the cells and centrifuged at 1000 g for 5 min. The absorbance of the supernatant was read at 540nm using distilled water as blank. The dilution of RBC suspension which gives a suitable absorbance for 100% hemolysis was selected.

The experiment was done with each concentrations of WAF as follows:

To 0.1 ml of the stock RBC suspension in a centrifuge tube, 5 ml of isotonic saline was added and incubated for 30 min at room temperature and then centrifuged at 1000 g for 5 min. The haemoglobin content of the clear supernatant was read at 540 nm. This gives absorbance of the 'blank' (B). Similarly 4.5 ml of distilled water was added to 0.1 ml of the stock RBC suspension in a centrifuge tube and incubated for 30 min at room temperature. Then 0.5 ml of WAF was added in such a way that the final WAF concentrations were 1.5ppm, 3.0ppm or 5.0ppm. Then it was centrifuged at 1000g for 5 min and the absorbance of the clear supernatant was measured at 540nm. This gives the absorbance corresponding to 100% hemolysis (H). In the third set 4.0 ml of hypotonic saline and 0.5 ml of distilled water were added to 0.1 ml of the stock RBC suspension in a centrifuge tube and then incubated for 30 min at room temperature. Then 0.5 ml of the WAF

was added in such a way that the final WAF concentrations were 1.5ppm, 3.0ppm or 5.0ppm. Then it was immediately centrifuged at 1000g for 5 min and the absorbance of the supernatant was read at 540nm. This gives the absorbance of the 'control'(C). To the fourth set 4.0ml of hypotonic saline was added to 0.1ml of the stock RBC suspension in a centrifuge tube. Then 0.5ml of WAF was added in such a way that the final WAF concentration in the tubes were 1.5ppm, 3.0ppm or 5.0ppm and incubated for 30 min at room temperature. After incubation 0.5ml of distilled water was added and centrifuged at 1000g for 5 min. The absorbance of the supernatant was read at 540nm and this gives the absorbance corresponding to 'test' (T).

**Calculations**

% Hemolysis in the control (X) = (C-B / H-B) x 100

% Hemolysis in the test (Y) = (T-B / H-B) x 100

% Labilisation by test = (Y-X / X) x 100

**b. Estimation of Haemoglobin (Cyanmethaemoglobin method)**

Blood was drawn from the cardinal vein in plastic syringes and used for the various assays.

**Procedure**

0.02 ml of blood was mixed with 5 ml of Drabkins diluent solution and allowed to stand for 5 min for the formation of Cyanmethaemoglobin. Absorbance was measured against a reagent blank which consisted of 5 ml of diluent solution. Using cyanmethaemoglobin standard, a standard

calibration curve was prepared from which the values of haemoglobin can be read directly as g / dl.

**c. Counting of Red Blood Corpuscles (RBC)**

**Procedure**

RBC count was done with a Neubauer crystalline counting chamber as described by Sohn and Henry (1969). The blood was collected in a vial containing 2% ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The blood was drawn up to 0.5 marks in RBC pipette and immediately the diluting fluid is drawn up to the mark 101 (thus the dilution is 1: 200). The solution was mixed well by shaking gently. It was allowed to stand for 2 minutes. The counting chamber and cover glass were cleaned and the cover glass was placed over the ruled area. Again the solution was mixed gently and the stemful of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle 40° It was allowed to stand for 2 to 3 minutes for RBC's to settle. Afterwards the ruled area of the counting was focused under the microscope and the number of RBC's were counted in five small squares of the RBC column under high power and the number of RBC's per cubic mm were calculated accordingly

$$\frac{\text{No. of cells} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Area counted}}$$

**d. Counting of White Blood Corpuscles (WBC)**

**Procedure**

WBC count was done as per the procedure described by Donald Hunter and Bomford (1963). Blood was collected in vial containing 2% EDTA

as an anticoagulant. The blood was drawn up to 0.5 marks of WBC pipette and immediately the diluting fluid is drawn up to the 11 mark above the bulb (The dilution fluid consists of 1.5ml of glacial acetic acid and one ml of aqueous Gentian violet solution made up to 100ml with distilled water). The solution was mixed thoroughly by shaking gently It was allowed to stand for 2 to 3 minutes. The Neubauer counting chamber and cover glass were cleaned and the cover glass was placed over the ruled area. Again the solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle 40° It was allowed to stand for 2 to 3 minutes and the WBCs were counted in the 4 corner square millimeters. The number of WBCs per cubic millimeter was calculated accordingly

$$\frac{\text{No. of cells} \times \text{Dilution factor} \times \text{Depth factor}}{\text{Area counted}}$$

**e. Determination of Packed Cell Volume (PCV) / Haematocrit value (Microhaematocrit centrifugation method)**

**Procedure**

From the cardinal vein blood was collected in EDTA tube and it was allowed to run about ½ to ¾th length of heparinized even bored capillary tubes and the tubes were sealed on the opposite end using sealing wax. The tubes were then transferred to a high speed microhaematocrit centrifuge and placed in the grooves of the capillary head. They were centrifuged for 15 min at 12,000 rpm. PCV was measured directly on a microhaematocrit reader associated with the centrifuge as volume percent.

## 5.2C. RESULTS

Experimental results of effect of different concentrations of WAF exposure on erythrocyte membrane stability in *O.mossambicus* was given in Table 5.2.1 and in Figure 5.2.1. The results obtained from three different concentrations of WAF exposure for 21 days were analysed statistically using one-way ANOVA of the raw data, followed by LSD analysis.

**Table 5.2.1 Percentage hemolysis in *O.mossambicus* on exposure to different concentrations of WAF *in vitro***

| Group | % Hemolysis |
|---|---|
| Control | 0 |
| 1.5ppm | 18.41 ± 1.07 |
| 3.0ppm | 35.82 ± 1.14 |
| 5.0ppm | 46.11 ± 2.08 |

⇒ Average of six values in each groups ± SD.

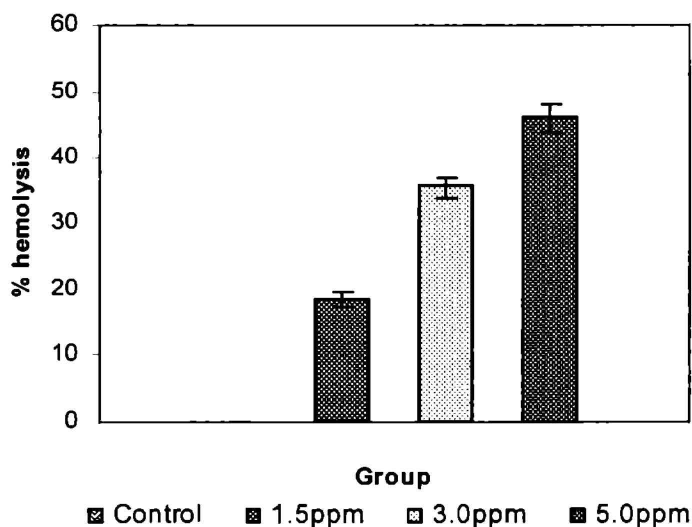



**Figure 5.2.1 Percentage hemolysis in *O.mossambicus* on exposure to different concentrations of WAF *in vitro***

One way ANOVA (Table 5.2.1 a) revealed that there was an overall significant change (P<0.001) between different concentrations of WAF exposure to that of control. As the concentration of WAF exposure increases, the labilisation of erythrocyte membrane also increases. 5.0ppm WAF treated experimental animals showed highest labilisation effect. Minimum effect was seen in 1.5ppm treated group.

**Table 5.2.1 a ANOVA for RBC membrane stability *in vitro***

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 10815.76 | 3 | 3605.253 | 2164.862 | 2.75E-25 | 3.098393 |
| Within concentrations | 33.307 | 20 | 1.66535 | | | |
| Total | 10849.06 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

Subsequent comparisons by multiple comparison (LSD) revealed that haemolytic effects of all the three concentrations of WAF exposure were significantly different (P<0.001) from control and also from one another (Table 5.2.1 b).

**Table 5.2.1 b Results of LSD analysis for *in vitro* studies**

| Groups | P value |
|---|---|
| Control Vs 1.5ppm | P < 0.001 |
| Control Vs 3.0ppm | P < 0.001 |
| Control Vs 5.0ppm | P < 0.001 |
| 1.5 ppm Vs 3.0ppm | P < 0.001 |
| 3.0ppm Vs 5.0ppm | P < 0.001 |
| 1.5ppm Vs 5.0ppm | P < 0.001 |

Results of Haemoglobin, WBC, RBC and PCV levels in the blood are given in the Table 5.2.2. The statistical significance of the experimental results was tested by one way ANOVA followed by LSD analysis.

**Table 5.2.2 Effect of different concentrations of WAF exposure on haematological parameters in *O.mossambicus***

| PARAMETERS | CONTROL | 1.5ppm | 3.0ppm | 5.0ppm |
|---|---|---|---|---|
| Hb | 9.85 ± 0.63 | 9.70 ± 0.14 | 8.75 ± 0.23 | 7.24 ± 0.10 |
| RBC | 2.78 ± 0.02 | 2.72 ± 0.09 | 1.96 ± 0.09 | 1.65 ± 0.01 |
| WBC | 2.91 ± 0.04 | 2.33 ± 0.07 | 2.13 ± 0.08 | 1.80 ± 0.02 |
| PCV | 17.25 ± 0.42 | 17 17 ± 0.15 | 19.24 ± 0.11 | 23.85 ± 0.14 |

⇒ Average of six values in each groups ± SD

**Table 5.2.2 a ANOVA for Hb**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 25.9182 | 3 | 8.6394 | 1024 | 4.92E-22 | 3.098393 |
| Within concentrations | 0.1694 | 20 | 0.00847 | | | |
| Total | 26.0876 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.2.2 b ANOVA for WBC**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 3.91005 | 3 | 1.30335 | 172.1731 | 1.88E-14 | 3.098393 |
| Within concentrations | 0.1514 | 20 | 0.00757 | | | |
| Total | 4.06145 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.2.2 c ANOVA for RBC**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 5.65725 | 3 | 1.88575 | 134.3127 | 2.02E-13 | 3.098393 |
| Within concentrations | 0.2808 | 20 | 0.01404 | | | |
| Total | 5.93805 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

**Table 5.2.2 d ANOVA for PCV**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 176.5288 | 3 | 58.84295 | 2142.861 | 3.04E-25 | 3.098393 |
| Within concentrations | 0.5492 | 20 | 0.02746 | | | |
| Total | 177.078 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

There was an overall significant variation (P<0.001) in the level of Haemoglobin, WBC, RBC and haematocrit values (Tables 5.2.2 a to 5.2.2 d). Subsequent comparisons by multiple comparison (LSD) (Table 5.2.2 e) revealed that Hb and WBC counts of all the three doses of WAF exposure were significantly different (P<0.001) from control and also from one another.

However, there was no significant change was observed in RBC counts and haematocrit value between control and 1.5ppm WAF treated groups. But there was a significant difference (P<0.001) was observed between 3.0ppm as well as 5.0ppm WAF treated groups with the control and also from one another (Table 5.2.2 e).

**Table 5.2.2e Results of LSD analysis for Haemoglobin, WBC, RBC and haematocrit in *O.mossambicus***

| GROUPS | PARAMETERS | | | |
|---|---|---|---|---|
| | Hb | WBC | RBC | Haematocrit |
| Control Vs 1.5ppm | P < 0.01 | P < 0.001 | NS | NS |
| Control Vs 3.0ppm | P < 0.001 | P < 0.001 | P < 0.05 | P < 0.001 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |
| 1.5 ppm Vs 3.0ppm | P < 0.05 | P < 0.001 | P < 0.05 | P < 0.001 |
| 3.0ppm Vs 5.0ppm | P < 0.001 | P < 0.05 | P < 0.05 | P < 0.05 |
| 1.5ppm Vs 5.0ppm | P < 0.001 | P < 0.001 | P < 0.001 | P < 0.001 |

NS-Not significant

## 5.2D. DISCUSSION

The present experimental results show that the erythrocyte membrane is maximally damaged and rendered leaky and spills its haemoglobin content at a higher concentration of WAF exposure. In support of our data Leighton *et al.* (1985) also observed hemolysis in blood samples of *herring gulls*, which was exposed to Prudhoe Bay crude oil. The first reason for the effect of petroleum hydrocarbons on the lability of erythrocyte membrane is the change in cation concentrations, which seems likely to reflect increased erythrocyte fragility and resultant erythrocyte haemolysis induced by uptake of petroleum hydrocarbons (Alkindi *et al.,* 1996). Another reason for the effect is that of the role played by peroxidation of the membrane lipids. Erythrocytes are prone to oxidative damage due to their high content of polyunsaturated fatty acids in the membrane, in combination with active metalloprotein- haemoglobin, which can function as an oxidase

and a peroxidase. High oxygen tension in many areas of the circulation due to crude oil impact, favors reactive oxygen species formation and membrane proteins and other functional proteins are cross-linked (Bainy *et al.*, 1996; Falconi *et al.*, 1987 Stern, 1985)

The oxidative denaturation of the erythrocyte membrane is considered another major cause of hemolytic process involving lipid peroxidation of RBC membrane (Caprari *et al.*, 1995; Falconi *et al.*, 1987). Polycyclic aromatic hydrocarbons of crude oil are a possible agent which damages the erythrocyte membrane (Leighton *et al.*, 1985) by increasing the oxidation of haemoglobin to methaemoglobin and / or inhibiting the reducing system responsible for the reverse reaction. Lipid peroxidation of erythrocyte membrane also causes them to lose their ability to change shape and squeeze through the smallest capillaries, and it will eventually lead to hemolysis (McCloskey and Oris, 1991).

It was observed that Hb content, RBC count and WBC count were significantly decreased by different concentrations of WAF of crude oil exposure and the maximum decline was noted in 5.0ppm dosed experimental animals. The decrease in Hb content and RBC counts under WAF exposure might be due to the prevalence of hypochromic microcytic anaemia, which is attributed to a deficiency of iron content and consequent decrease in Hb synthesis (Bhai *et al.*, 1971). A more plausible explanation is that the anaemia is due to a decreased rate of production of and /or to an increased loss or destruction of red blood cells. Anaemia is often due to an increase of plasma volume caused by disturbed water balance. The present

result is supported by the work of Klyszejko *et al.* (1982), who chronically exposed *Anguilla (L.)* to WAF of crude oil, showed lower Hb levels and lower numbers of RBC than the unexposed ones. WAF-induced blood anaemia has also been observed in experiments with fresh water cat fish, *Heteropneustes fossilis* (Prasad *et al.,* 1987; Omoregie, 1998).

Thus it may be inferred that both Hb and RBC are oxygen carrying devices and the quantitative decrease in their levels may lead to the derangement of the oxidative metabolism and is in consonance with a concomitant decrease in the tissue respiratory potentials mentioned earlier.

The results showed that the decrease in WBC count is found to be relatively more than that in RBC count, indicating that the capacity of the fish to withstand to any infection is diminished under crude oil impact; much more in the case of 5.0ppm WAF dosed fish. The decrease in WBC count suggests that the fish have decreased capacity to defend against microbial or bacterial infection. This decrease in the WBC count might be due to autolysis of the WBC, liberating the hydrolytic enzymes like phosphatases, pepsin, trypsin, lipase etc in the plasma (Wright, 1960; Wlasow and Dabrowska, 1990). In agreement with our findings a similar decrease in WBC was reported in rainbow trout (Wlasow, 1985) and in Nile tilapia (Omoregie, 1998). The drop in leucocyte count may lead to a decrease in the non-specific immunity of affected fish. Small lymphocytes are active in the increase, build-up and transport of globulins. A significant decrease in leucocyte numbers and particularly of small lymphocytes in fish may cause a decrease in antibody production and thus a reduced resistance to disease (Peters, 1986).

Haematocrit level was significantly increased in experimental animals exposed to WAF of crude oil and the maximum increase was noted in 5.0ppm dosed fish. Changes in the haematocrit value are closely connected with changes in erythrocyte volume. In agreement with our result, a significant increase in haematocrit value of cunnar was found, after toxic exposure to petroleum hydrocarbons for two weeks (Kiceniuk *et al.,* 1980). Gill damage associated with WAF of crude oil exposure (Chapter 7 1) and the inhibition of branchial ATPase enzymes bound to the gills could (Chapter 6.1) result in extreme osmoregulatory stress in the fish. Gill damage would most likely produce hypoxic conditions within the blood of fish (Oris and Giesy, 1985). These combined osmoregulatory stress and hypoxic conditions in the blood can result in swelling of red blood cells, explaining the increased haematocrit and decreased haemoglobin concentration found in the present study. Exposure to the petroleum hydrocarbons elicited a rapid and highly significant drop in the oxygen content (Val and Almedia-Val, 1999). Catecholamines especially noradrenaline have been reported to be released in response to conditions which give rise to hypoxaemia (Thomas and Perry, 1992).

Increased noradrenaline is likely to have been responsible for the significant increase in blood haematocrit (Zbanyszek and Smith 1994; Poirier 1996; Davison *et al.,* 1993). Thus it may be inferred that the dramatic decline in blood oxygen content of fish may further influence thyroid function and is likely to have triggered the significant increase in noradrenaline concentrations. Rising noradrenaline concentrations may trigger release of more erythrocytes including immature erythrocytes of larger size, initially elevating haematocrit (Alkindi *et al.*, 1996).

Thus it may conclude that petroleum hydrocarbons can significantly alters the haematological parameters of fish and also it can even cause an increased disintegration of erythrocytes. Further more, it induces the rupture of erythrocyte membrane and release of haemoglobin.

# Chapter 6

## Effects Of Petroleum Hydrocarbons On Branchial ATPase And Bioaccumulation Of Petroleum Hydrocarbons

## 6.1 STUDIES ON BRANCHIAL ATPase

### 6.1A. INTRODUCTION

Most aquatic animal cells maintain a high intracellular $K^+$ concentration and a low $Na^+$ concentration. The surrounding interstitial fluid has high $Na^+$ and low $K^+$ concentrations. This is accompanied by movement of $Na^+$ and $K^+$ across the cellular membranes against an electrochemical gradient by an active transport (energy-requiring) process. The active transport mechanism involving $Mg^{2+}$-dependent-$Na^+$-and $K^+$-activated adenosine triphosphatase ($Na^+$-$K^+$ ATPase) provides the largest contribution to the maintenance of $Na^+$ and $K^+$ transmembrane gradients (Trachtenberg *et al.*, 1981). Maintaining this $Na^+$ and $K^+$ gradient has a central role in, or is required for, the uptake by cells of metabolites such as glucose and aminoacids, regeneration of transmembrane potential during nerve excitation, transmembrane movement of $Ca^{2+}$ during muscle stimulation, maintenance of osmotic equilibrium in cells and control of transcellular ion movement (Haya and Waiwood, 1980; Morris and Edwards, 1995).

$Na^+$-$K^+$ATPase is an intrinsic membrane-bound protein which hydrolyzes ATP to ADP and inorganic phosphate and, in the process, makes the energy from ATP available for active cation transport (Schuurmans and Bonting, 1981). Harris and Bayliss (1988) reported that potential $Na^+$ pumping capacity of the gills was highly correlated with gill $Na^+$-$K^+$ATPase specific activities and more closely correlated with enzyme activity than $Na^+$ gradient. The $Na^+$-$K^+$ATPase enzyme has a role in the branchial uptake of sodium ions. The teleost fresh water fishes engage in active ion uptake to

maintain homeostasis. In fish, the uptake of $Ca^{2+}$ $Cl^-$ and $Na^+$ from the water has largely been attributed to mitochondria-rich cells, the so-called chloride or ionocytes (McCormick, 1995; Li *et al.,* 1995; Perry, 1997). These cells contain the bulk of the branchial $Na^+$-$K^+$ATPase, the driving force for branchial $Na^+$ handling, which is located in their extensive tubular membrane system. The activity of $Na^+$-$K^+$ATPase is also the driving force for a secondary mechanism, the branchial $Na^+/Ca^{2+}$ exchanges, which plays a crucial role in $Ca^{2+}$ transport and homeostasis of these cells (Benders *et al.,* 1994; Verbost *et al.,* 1994; Flik *et al.,* 1995). Studies with isolated opercular membrane (McCormick, 1990; Wendelaar Bonga *et al.,* 1990) of fresh water fish suggest that the activity of $Na^+$-$K^+$ATPase is directly correlated with the chloride cell density.

Towle (1981) observed that $Na^+$-$K^+$ATPase plays a central role in whole body ion regulation. Thus, any toxicant that interferes with ionic homeostasis may exhibit an altered $Na^+$-$K^+$ATPase activity at sublethal concentrations. Xenobiotics can interact directly with the enzyme or alter $Na^+$-$K^+$ATPase activity due to disruption of energy producing metabolic pathway (Watson and Beamish, 1980). ATP stores were reduced by increased energy demand in response to crude oil exposure (Omoregie, 2002). Since $Na^+$-$K^+$ATPase turnover rate is extremely rapid (Hossler, 1980), the enzyme would be sensitive to factors affecting protein biosynthetic pathways. Altered $Na^+$-$K^+$ATPase activity can arise in response to a wide variety of toxicants including crude oil. To maintain ionic homeostasis during exposure to pollutants, fish activate a number of biochemical and

physiological processes aimed at detoxifying (Roesijadi, 1996) and restoring the branchial ion uptake machinery (McDonald and Wood, 1993).

This study was undertaken to investigate the influence of different concentrations of water-accommodated fractions of crude oil on biochemical ion transport mechanisms, in particular the ion-dependent $Na^+$-$K^+$ATPase and $Mg^{2+}$ATPase of gills of *O.mossambicus* because gills are the major osmoregulatory organ whether the animals is in hypo or hyper osmoregulator or an osmoconformer. The major amount of $Na^+$-$K^+$ATPase of the teleost and probably vertebrate gill are found in the basolateral membrane of chloride cells and therefore in close contact with the blood, interstitial fluid, and external water (Towle, 1981). In addition, gill epithelium comprises approximately 95% of the body surface of the fish (Parry, 1966), is relatively permeable to water, and is the main site of osmotic water movement. Since, for most pollutants including crude oil, uptake from water is the most important route, gills are a primary target organ and may be one of the first organs to exhibit symptoms of sublethal toxicity.

## 6.1B. MATERIALS AND METHODS

Collection, acclimation of fish, method of WAF preparation and method of dosing were the same as that described in Chapter 2B. Different concentrations of WAF were dosed for three weeks to the experimental animals and then the gill tissues from control and WAF treated groups was dissected out. Then the gill tissues were washed in ice-cold 0.33M sucrose and then 10% of gill homogenate was prepared in ice-cold 0.33M sucrose solution.

**a. Extraction of the enzyme ($Na^+$-$K^+$ATPase)**

10% gill homogenate was centrifuged at 3000g for 15 min and the supernatant obtained was again centrifuged in a cold refrigerated centrifuge at 12000g for 30 min. The clear supernatant was again centrifuged at 35000g for 30 min. The pellet so obtained corresponds to the heavy microsomal fraction (Davis, 1970). The pellet was then resuspended in cold 0.33M sucrose and used as the enzyme source.

**b. Assay for $Na^+K^+$ATPase (ATP Phosphohydrolase E.C. 3.6.1.3)**

$Na^+$-$K^+$ activated, $Mg^{2+}$ dependent ATPase (Total ATPase) was determined by using the reaction mixture containing 60mM NaCl, 20mM KCl, 2mM $MgCl_2$, 30mM Tris-HCl (pH 7.5) and 2.5mM Tris-ATP The $Mg^{2+}$ATPase was measured by substituting sucrose in place of NaCl and KCl. The reaction mixture was incubated at 37°C for 15 min. The $Na^+$-$K^+$ATPase activity was calculated in terms of the difference between total and $Mg^{2+}$ATPase values. After incubation 2 ml of 10% Trichloro acetic acid was added to the reaction mixture and the supernatant separated off by centrifugation at 1300 g for 10 min. The inorganic phosphate liberated from ATP was estimated by the method of Fiske and Subbarow (1925). The specific activity of $Na^+$-$K^+$ATPase was defined as micromoles of Pi / mg of enzyme protein / h. Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

## 6.1C. RESULTS

Branchial ATPase activities are given in Table 6.1.1 and Figures 6.1.1 and 6.1.2.

**Table 6.1.1 Effect of different concentrations of WAF exposure on the gill $Na^{+}$-$K^{+}$ ATPase and $Mg^{2+}$ATPase activities in *O.mossambicus.***

| GROUP | TOTAL ATPase | $Na^{+}$-$K^{+}$ ATPase | $Mg^{2+}$ ATPase |
|---|---|---|---|
| Control | 52.72 ± 1.16 | 28.46 ± 1.47 | 24.24 ± 1.77 |
| 1.5ppm | 43.38 ± 1.33 | 23.86 ± 1.39 | 19.51 ± 1.18 |
| 3.0ppm | 38.44 ± 1.51 | 20.13 ± 1.08 | 18.31 ± 1.32 |
| 5.0ppm | 30.79 ± 1.35 | 15.84 ± 1.68 | 14.95 ± 1.48 |

⇒ Activities of ATPase are expressed as μ moles of inorganic phosphate (Pi) liberated / h / mg protein.

⇒ Average of six values in each groups ± SD.

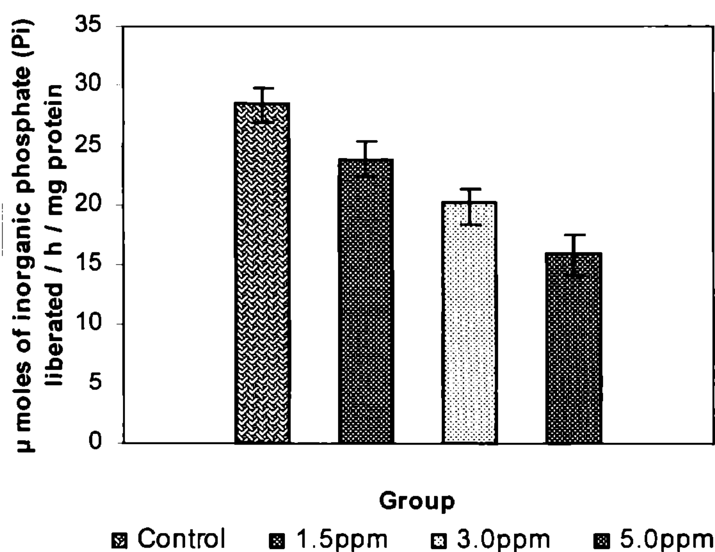



**Figure 6.1.1 $Na^{+}$-$K^{+}$ATPase activity in *O. mossambicus.***

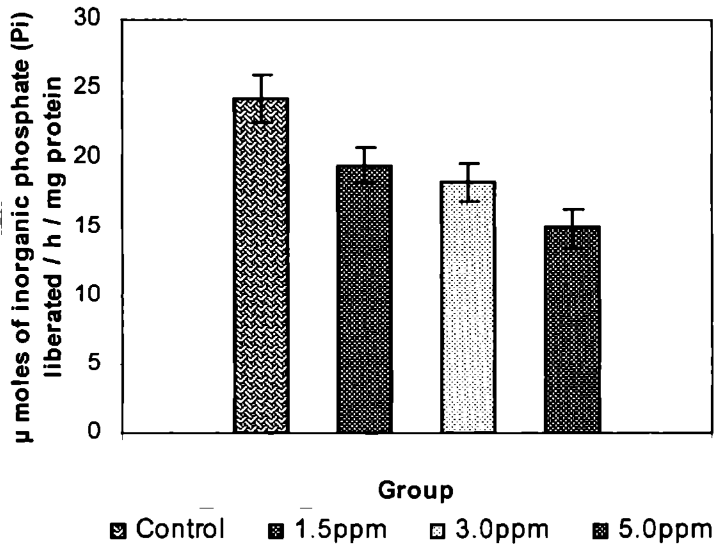



**Figure 6.1.2 $Mg^{2+}$ATPase activity in *O. mossambicus***

**Table 6.1.1 a ANOVA for branchial $Na^{+}$-$K^{+}$ATPase**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 519.6403 | 3 | 173.2134 | 134.2465 | 2.03E-13 | 3.098393 |
| Within concentrations | 25.80528 | 20 | 1.290264 | | | |
| Total | 545.4456 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

One way Analysis (Table 6.1.1 a) revealed that there was an overall significant change in the activity of branchial Na+-K+ATPase in presence of WAF. Further analysis by LSD (Table 6.1.1 c) showed that there was a significant decrease ($P<0.001$) in all the WAF treated groups when compared with the control group. Comparison between different concentrations also showed significant change.

**Table 6.1.1 b ANOVA for branchial $Mg^{2+}$ATPase**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 266.0477 | 3 | 88.6825 | 51.30 | 1.42E-09 | 3.098393 |
| Within concentrations | 34.5706 | 20 | 1.72853 | | | |
| Total | 300.6183 | 23 | | | | |

SS-Sum of squares, df-degrees of freedom, MS -mean of squares.

One way analysis of variance revealed that there was an overall significant change (P<0.001) in branchial Mg2+ATPase (Table 6.1.1 b) in all the WAF treated animals. Subsequent comparison by LSD analysis (Table 6.1.1 c) revealed that there was a significant inhibition of branchial Mg2+ATPase in three different WAF treated experimental animals when compared to control group. LSD value at 5% level was 1.586. Comparisons between different concentrations also showed significant change. 5.0ppm WAF treated animals showed maximum total ATPase inhibition, which had followed by 3 0ppm treated and finally by 1.5ppm treated groups. This revealed that as the concentration of WAF exposure increases, it causes greater damage to gill membrane of the WAF treated animals.

**Table 6.1.1 c Results of LSD analysis for ATPases**

| Groups | $Na^{+}$-$K^{+}$ATPase | $Mg^{2+}$ATPase |
|---|---|---|
| Control Vs 1.5ppm | P < 0.001 | P < 0.001 |
| Control Vs 3.0ppm | P < 0.001 | P < 0.001 |
| Control Vs 5.0ppm | P < 0.001 | P < 0.001 |
| 3.0ppm Vs 1.5ppm | P < 0.001 | P < 0.05 |
| 5.0ppm Vs 3.0ppm | P < 0.001 | P < 0.001 |
| 5.0ppm Vs 1.5ppm | P < 0.001 | P < 0.001 |

## 6.1D. DISCUSSION

A significant inhibition of $Na^+$-$K^+$ATPase and $Mg^{2+}$ATPase activity was observed in gill tissues of *O.mossambicus* when exposed to 1.5ppm, 3.0ppm and 5.0ppm of WAF of Bombay High crude oil for 3 weeks. The results revealed that inhibition of $Na^+$-$K^+$ATPase and $Mg^{2+}$ATPase activity by WAF of crude oil seriously affect their contribution to transepithelial $Na^+$ transport, disturbs $Na^+$ homeostasis of the body as well as inhibit the 'household' function of $Na^+$-$K^+$ATPase (maintenance of intracellular ion homeostasis) (Quabius *et al.,* 1998).

Branchial $Na^+$-$K^+$ATPase, a key enzyme in branchial $Na^+$ uptake, is a primary target for crude oil. Previous investigations in saltwater and freshwater acclimated rainbow trout (*Salmo gairdneri)* have revealed a very similar inhibitory effect of $Na^+$-$K^+$ATPase when exposed to crude oil as a water-soluble fraction and as dispersion (Wong and Engelhardt, 1984).

Thurberg *et al.*(1978), working on fish from the Argo Merchant fuel oil spill area reported reduced plasma sodium ion concentration in yellowtail flounder and haddock from the oil-exposed area. They also found reduced potassium ion level in yellowtail, winter flounder, and haddock and reduced plasma osmolality in yellowtail and haddock from the exposed area. Brauner *et al.* (1999) reported that Urucu crude oil affects ion regulation in facultative air breather, *Hoplosternum littorale* when exposed to water-soluble fraction of the Urucu crude oil. They also reported that there was a significant reduction in plasma $Na^+$ and $K^+$ after crude oil exposure.

Previous studies on exposure of bluegill sunfish to anthracene clearly demonstrated that the decrease of specific activity of branchial $Na^+$-$K^+$ATPase impaired kinetics of $Na^+$ transport leading to reduced plasma $Na^+$ levels in these animals (McCloskey and Oris, 1993). The results of the present study with *O mossambicus* exposed to WAF of crude oil is in agreement with this observation as it demonstrates a dose-dependent decrease in the specific activities of the gill $Na^+$-$K^+$ATPase and $Mg^{2+}$ATPase.

The inhibitory effect on $Na^+$-$K^+$ATPase is generally ascribed to structural and functional damage to the gills as a result of accumulation of toxicant (Pelgrom *et al.,* 1995). Lipid peroxidation is a highly destructive process and alters the structure and function of cellular membrane (Kale and Sitaswad *et al.,* 1990). Disrupted tissues are known to undergo lipid peroxidation at a faster rate than normal ones (Anjali and Kala, 2001). Peroxidation brings about change in structure and inactivates a number of membrane-bound enzymes and protein receptors (Maridonneau *et al.,* 1983; Yahawa and Nagatuka, 1983) induces alterations of respiratory functions and causes loss of-SH groups from the membrane-bound proteins (Leyko and Bartosz, 1986). Integrity of membrane is essential to maintain cation homeostasis. In WAF exposed systems, as mentioned earlier, membranes are critical targets of detrimental effects (Srivastava *et al.,* 1998). $Na^+$-$K^+$ATPase is an intrinsic membrane-bound protein and required specific lipids for its activity Therefore it may be inferred that WAF-induced damage to the cell membrane lipids and proteins resulted in significant decrease of branchial ATPase activity. The damage via peroxidation can also disrupt branchial membrane integrity (Sato, 1987).

At the light microscopical level, structure effects of crude oil exposure on the gills have been reviewed by Khan (1995); Haensly *et al.* (1982) and Solangi, (1982). From the present histopathological study it is evident that when petroleum hydrocarbons have accumulated above a critical level, it can damage the gill tissue extensively. The present experimental results exhibit marked increase of cell degeneration, dilation of intercellular spaces and necrosis representing accidental and programmed cell death. It was observed during the experimental period that, as the concentration of WAF of crude oil increased, the severity of necrosis also increased (Chapter7). Gill damage associated with WAF and the inhibition of ATPase enzyme bound to the gills could result in extreme ormoregulatory stress in *O mossambicus*. Gill damage would most likely produce hypoxic conditions within the blood of fish (Oris and Giesy, 1985). The combined osmoregulatory stress and hypoxic conditions in the blood can result in swelling of red blood cells, explaining the increased haematocrit and decreased haemoglobin concentration found in the present study. The findings from the present study suggest that a general disruption of cellular membrane function is an important mechanism of acute toxic of action in fish exposed to WAF of crude oil. Evidence of hemolysis was observed in the blood of fish exposed to WAF of crude oil suggesting that the binding of aromatic hydrocarbons in WAF of crude oil to cellular membranes (Leighton *et al.*, 1985) leads to the WAF-mediated disruption of RBC membranes. Cellular membranes are further implicated due to the fact that the membrane-bound ATPase enzyme was inhibited. Disruption of membrane function is also supported by the extreme gill and dorsal epidermal damage observed in

bluegill sunfish exposed to anthracene, which is one of the components of WAF of crude oil (Oris and Giesy, 1985).

Cortisol, the main teleost glucocorticoid in fish (Balm *et al.*, 1989; Pottinger *et al.*, 1994) plays a crucial role in the acclimation processes of the gills to external factors affecting branchial ionoregulatory functions. Some studies suggest that fish exposed to petroleum hydrocarbons have elevated concentrations of plasma cortisol (Engelhardt *et al.*, 1981). In chronic stress, elevated cortisol is suggested to increase the adrenergic responsiveness of the hepatocytes (Reid *et al.*, 1992). Works of Alkindi *et al.* (1996) on Flounders, *Pleuronectes flesus* and Brauner *et al.* (1999) on *Hoplosternum littorale* showed that there was an increase in plasma cortisol concentrations when the experimental animals were exposed to water-soluble fractions of Omani crude oil. It has also been observed that the stress hormone like cortisol *in vivo* induced a dose-dependent increase in apopstosis of chloride cells and pavement cells, thus interfering with branchial ATPase and ionic homeostasis (McCormick, 1995).

From the foregoing results it is evident that WAF of Bombay High crude oil inhibits the branchial ATPase activity by disrupting the integrity of cell membrane and function of blood and gills as well as increasing the permeability of branchial cells to aromatic hydrocarbons of WAF via enhanced cortisol activity; thereby the fish experiences extreme osmoregulatory stress.

## 6.2. STUDIES ON BIOACCUMULATION OF PETROLEUM HYDROCARBONS

### 6.2A. INTRODUCTION

The process of accumulation and retention of a number of environmentally available materials by organisms provide an excellent means of screening the environment. Among these materials, petroleum hydrocarbons are of particular significance (Klosterhaus *et al.,* 2002). It is possible to detect pollutants in organisms even when the concentrations in the water are too low or too variable to be determined by routine analytical tools. The process of bioaccumulation has attracted the environmentalists because of the following additional advantages it offers. It gives direct indication of pollution and may also have important influences on food chains. Moreover, it provides some easily measurable indication of the ecological consequences of pollution (Ferguson and Chandler, 1998).

Fishes are good bioaccumulators of petroleum hydrocarbons. The lipophilic character of petroleum hydrocarbons facilitates their speedy accumulation in organisms (Parrish *et al.,* 1989). The presence of polycyclic aromatic hydrocarbons in the tissues of a wide variety of fresh water and marine organisms strongly indicates that these organisms are able to accumulate polynuclear aromatic hydrocarbons present at low concentrations in the ambient media, food or sediments (Xu *et al.,* 1998). Ingestion of contaminated sediment and polycyclic aromatic hydrocarbon contaminated water in sediment are major routes of entry for petroleum hydrocarbons in the body for benthic animals (Varanasi *et al.,* 1989). Thomas *et al.,* (1999) studied that mussels, *Mytilus troussuls* collected from the beaches in Prince

William Sound which had been impacted by the Exxon Valdez oil spill showed a significant increase of total polynuclear aromatic hydrocarbons in its tissues. Studies of Ray *et al.* (1990) with *Clarias batrachus* have pointed out that monitoring bioaccumulation in specific tissue provides a better basis than whole body analysis. In the present study- liver, gill and muscle tissues of *O.mossambicus* exposed to 1.5ppm, 3.0ppm and 5.0ppm of WAF for 21 days was selected and monitored for the extent of bioaccumulation. Since liver being the metabolic centre involved in anabolic and catabolic activities; gills being the main route of entry of petroleum hydrocarbons and muscle being the major tissue that ends up as food for human use, they are selected for the current study.

## 6.2B. MATERIALS AND METHODS

Experimental design was the same as that described in Chapter 2B.

### a. Extraction of petroleum hydrocarbons (PHC) from tissues

PHC content in the gill, liver and muscle tissues of *O.mossambicus* was estimated by the method of Donkin and Evans (1984) with slight modification.

Extraction of petroleum hydrocarbons was conducted using a steam distillation apparatus. 3.0g of pooled gill, liver and muscle tissues of control and different concentrations of WAF treated animals were weighed separately and homogenized in n-hexane and transferred into four different round bottomed flasks containing 5ml of 4M Sodium hydroxide solution, 15ml n-hexane (HPLC grade) and 50ml of distilled water each. The mixture was

saponified for 2 hours at a temperature of about 80°C and the resultant solution was neutralized by 20ml of 1M HCl and 10ml distilled water. The distillation was continued for another 2 hours. The apparatus was cooled to room temperature and the solvent collected in the water estimator was transferred to a clean vial. The vials were stoppered and stored in the deep freezer overnight. The n-hexane extracts were dried over anhydrous sodium sulphate and purified by activated alumina cleaned up columns. The petroleum hydrocarbons content of the samples were estimated using the fluorescence spectrophotometer (EX-310nm, EM-360nm). The values were expressed as µg of petroleum hydrocarbons / g wet wt. tissue.

**b. Statistical Analysis**

Statistical analysis of results was done by ANOVA followed by Least Significance Difference (LSD).

## 6.2C. RESULTS

Bioaccumulation of petroleum hydrocarbons in various tissues of *O.mossambicus*, on exposure to different concentrations of WAF for three weeks are given in Table 6.2.1 and in Figure 6.2.1

**Table 6.2.1 Tissue load of petroleum hydrocarbons in *O.mossambicus***

| GROUP | LIVER | GILL | MUSCLE |
|---|---|---|---|
| Control | 0 | 0 | 0 |
| 1.5ppm | 13.65 ± 0.29 | 23.68 ± 1.14 | 10.32 ± 0.39 |
| 3.0ppm | 26.89 ± 0.72 | 44.53 ± 1.82 | 27.41 ± 1.02 |
| 5.0ppm | 45.32 ± 1.28 | 58.43 ± 2.35 | 39.02 ± 1.46 |

⇒ Values are expressed as µ g of PHC / g wet wt. tissue.

⇒ Average of six values in each groups ± SD.

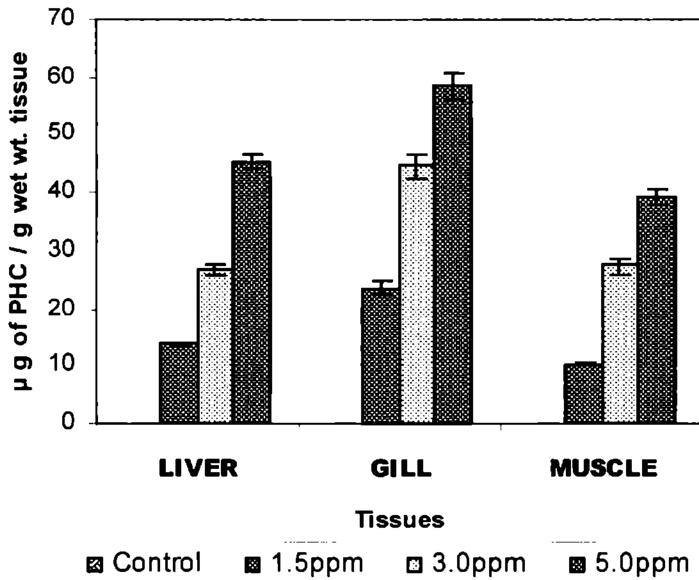



**Figure 6.2.1 Tissue load of PHC**

**Table 6.2.1 a Two-Factor ANOVA for PHC tissue load**

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between concentrations | 3834.925 | 3 | 1278.308 | 54.23316 | 9.71E-05 | 4.757055 |
| Between tissues | 352.9177 | 2 | 176.4589 | 7.486396 | 0.023415 | 5.143249 |
| Error | 141.4236 | 6 | 23.5706 | | | |
| Total | 4329.266 | 11 | | | | |

SS-Sum of squares, df-degrees of freedom, MS- mean of squares

Two-Factor ANOVA (Table 6.2.1 a) and subsequent comparison by LSD analysis (Table 6.2.1 b) revealed that there was a significant increase ($P<0.001$) between control and different concentrations of WAF treated groups. Comparisons between concentrations also revealed a significant change. LSD analysis also showed (Table 6.2.1 c) that different tissues differed significantly from one another ($P<0.05$) except between muscle and liver.

**Table 6.2.1 b Results of LSD analysis for PHC bioaccumulation**

| GROUPS | P Value |
| --- | --- |
| Control Vs 1.5ppm | $P < 0.001$ |
| Control Vs 3.0ppm | $P < 0.001$ |
| Control Vs 5.0ppm | $P < 0.001$ |
| 3.0ppm Vs 1.5ppm | $P < 0.05$ |
| 5.0ppm Vs 1.5ppm | $P < 0.001$ |
| 5.0ppm Vs 3.0ppm | $P < 0.05$ |

**Table 6.2.1c Results of LSD analysis for PHC bioaccumulation**

| GROUPS | P Value |
| --- | --- |
| Liver Vs Gill | $P < 0.05$ |
| Liver Vs Muscle | NS |
| Gill Vs Muscle | $P < 0.05$ |

## 6.2D. DISCUSSION

The results indicate that there is a significant accumulation of petroleum hydrocarbons in all the test tissues under the exposure of 1.5ppm, 3.0ppm and 5.0ppm of water-accommodated fractions of crude oil. Among the tissues petroleum hydrocarbons accumulation at 5.0ppm dosed animals was maximum in the gill followed by liver and muscle tissues. Hence it was inferred that even a small concentration of WAF of crude oil may cause greater accumulation of petroleum hydrocarbons in the tissues.

Amodio-Cocchieri *et al.* (1993) have reported that there was an increased concentration of polycyclic hydrocarbons in the tissues of common mussel collected from petroleum hydrocarbon-contaminated site. McCain *et*

*al.* (1990) have shown that juvenile Chinook salmon *(Oncorhyncus tshawytscha)* from an urban estuary in Seattle accumulate higher levels of aromatic hydrocarbons than the salmons from a rural estuary. Crustacean tissues sampled after crude oil spills registered paraffins and aromatics bioaccumulated from the contaminated environment: 11.8 g/g n-paraffins were detected in the barnacle, *Mitella polymerus* (Clark *et al.*, 1973), 7-11 (g/g aromatics in the crab, *Cancer irroratus* and 103-130 g/g aromatics in the gut, 15-230 g/g aromatics in the stomach, 2-3 g/g aromatics in the claw muscle and 1-4 (g/g aromatics in the abdominal muscle of the lobster, *Homarus americanus* making the digestive tract tissues the most prone to bioaccumulation of petroleum hydrocarbons in crustaceans (Scarrat and Zitko, 1972). Bender *et al.* (1988) in their study on the relative accumulation of petroleum hydrocarbons in fishes and shell fish, calculated that oysters (*Crassostrea virginica)* accumulated about 3.7 times more total polycyclic aromatic hydrocarbons than did clams *(M. mercenaria and R.cuneata)* at 25°C. It was also reported that certain compounds like phenanthrene and acenaphthalene in crude oil are strongly retained in lipid tissues of Asian clam *(Potamoeorbula amurensis)* and probably are only slowly depurated (Pereira *et al.*, 1992). All these above observations of different workers support the findings of present investigation.

It was also shown that bioaccumulation via particle ingestion depends on the feeding rate and feeding selectivity of the organism and the assimilation efficiency and the contaminant concentration of the ingested food particles (Lee *et al.*, 1990). Neff (1979) have reported that the uptake of aromatic hydrocarbons into the tissues of marine fish was found to be directly

related to the amount of aromatic hydrocarbons in the water-accommodated fractions of crude oil. Naphthalene is the most water soluble of the polycyclic aromatic hydrocarbons tested, rendering it more readily bioavailable.

Thus it is evident that fishes are good bioaccumulators of petroleum hydrocarbons and they concentrate the petroleum hydrocarbons to a level that makes them easier to detect in various tissues and it gives direct indication of pollution in the environment.

# *Chapter* 7

# Histopathology And Incidental Pathology

## 7.1 HISTOPATHOLOGY

### 7.1A. INTRODUCTION

The study of structural damage of organs or tissues is an integral part of pollution toxicology. Organisms in general and aquatic ones in particular are easily susceptible to the toxic effects of environmental contaminants. A clear understanding of the cause and effect of such toxic reactions could be identified only with the help of histopathology. Histopathological techniques are rapid, sensitive, reliable and comparatively inexpensive tools for the assessment of stress response to pollutants. Depending on the nature of the toxicant, some of the cellular changes could be specific whereas some could be of a general nature to toxicant stress (Moore, 1993).

The cell has a great capacity for adaptation and is able to respond to changes in the internal and external environments by alterations in both cellular structure and function. Cells which have reached their limit of adaptability begin to show structural changes which indicate their failure to withstand the changed environment. If adverse conditions persist or if the initial pathological stimulus was severe, then these processes continue and progress into a sequence of events leading to cell death (cell necrosis) (Varanasi, 1989; Menon, 1999). Thus, histological approaches and techniques are useful for studying changes in the structure of tissues and their composite cells, thus giving indications of the degree of stress and of the adaptive capability of the organism.

During the last decade, fish was used widely to indicate the degree of oil pollution. Woodward *et al.* (1983) and Prasad (1988) who studied the histopathological effects of petroleum hydrocarbons on fishes at the light microscopic level and observed that the changes associated with the gills were oedema, lesions and mucous cell hyperplasia. Chronic exposure of cod to 150-300ppb of Venezuelan crude and as little as 50ppb of Hibernia crude causes enlargement of the heart (Khan *et al.*, 1981). Livers in fish exposed to 100mg Louisiana whole crude oil and its water-soluble fraction per litre for 13 days showed slight necrosis of both acinar and hepatic cells and noticeable fatty vacuolation of hepatocytes (Solangi *et al.*, 1982). Fletcher *et al.* (1982) noted a reduction in the size of the testes in male *P americanus* exposed to oil sediments, the probable reason being the increased in metabolic demands of the fish by the oil, thereby requiring them to utilize the testes as an energy source.

The route that the petroleum hydrocarbon enters into the *O.mossambicus* often indicates the choice of organs for the histological examination. For the present histological study, gill tissue is selected because the major route of entry of petroleum hydrocarbons is through gills by virtue of its immediate contact with the medium containing water-accommodated fractions of crude oil; there is every likelihood of structural damage to occur in this tissue.

## 7.1B. MATERIALS AND METHODS

This part of thesis reports the histopathological effects on the gill tissues of *O.mossambicus* exposed to 1.5ppm, 3.0ppm and 5.0ppm

concentrations of WAF of crude oil for a period of 21 days in the laboratory conditions.

The details of the test animals, toxicant concentrations and preparation of exposure medium etc. have been given earlier in Chapter 2 B.

### a. PREPARATION OF MICROSLIDES

After termination of the exposure period for 3 weeks, the animals were dissected out and gill tissue was washed in running water and immediately fixed in the Bouin's fixative (Luna, 1968) for 24 h. The following time schedule was used to make paraffin wax blocks for histological studies.

1 Washed overnight in running water
2. The tissues were treated with saturated solution of lithium carbonate in 70% alcohol to remove yellow colour of picric acid.
3. After softening, the tissues were stored in fresh 70% alcohol. In this stage, the tissues can be stored till further processing.
4. Tissues dehydrated by transferring them sequentially to 80%, 90%, 95% alcohol for 2 h each.
5. Transferred to absolute alcohol (2 changes) for 1 h each.
6. Placed the tissues in 1:1 mixture of absolute alcohol and xylene for 30 min.
7 Cleared in xylene until the tissues became translucent.
8. Tissues transferred to a mixture of xylene and paraffin wax and left overnight.

9. Infiltered the tissues in 2-3 changes of molten paraffin wax of melting point 60-62°C for 1 h each.
10. Embedded in paraffin wax of melting point 60-62°C.

The blocks were trimmed and sections of 3-4 μm thickness were cut with a rotary microtome.

**b. STAINING TECHNIQUE FOLLOWED WITH HAEMATOXYLIN EOSIN STAIN**

The slides were transferred to xylene absolute alcohol (1:1) for 1 minute, hydrated by passing through a descending series (absolute, 90%, 80%, 70%, 50% and 30%) of alcohol for 1 minute each and washed the slides in a running tap water for 5 minutes and then the slides were stained in haematoxylin for 2 minutes and again washed the slides in running tap water for 3 minutes and dipped in acid absolute alcohol mixture (0.5:100) once and again washed in running tap water for 3 minutes and kept the slides for 1 minute in eosin for staining. The stained slides were then dehydrated by passing them through an ascending series-50%, 70%, 80%, 90%, 95% and absolute alcohol for 1 minute each and washed in running tap water for exactly once and slides were dried using filter paper and finally cleared in xylene and mounted in D. P X.

The sections were viewed and photographed under a light microscope at 40x magnifications.

## 7.1C. RESULTS

The following are the several alterations observed in gill tissue of *O.mossambicus* exposed to 1.5ppm, 3.0ppm and 5.0ppm concentrations of water-accommodated fractions of crude oil for three weeks.

The gill tissue of the control group had normal gill structure (Plate 1). The gills of *O.mossambicus* comprise two sets of four holobranchs, forming the sides of the pharynx. Each holobranch consists of two hemibranchs projecting from the posterior edge of the branchial arch or gill arch in such a way that the free edges diverge and touch those of the adjacent holobranchs. Close examination of the hemibranchs of a gill shows that they consist of a row of long thin filaments, the primary lamellae, which project from the arch like the teeth of a comb. The surface area of the primary lamellae is increased further by the formation of regular semi lunar folds across its dorsal and ventral surface- the secondary lamellae. The dorsal and ventral rows of secondary lamellae on each primary are staggered so that they complement the spaces in the rows of lamellae of adjacent filaments.

- Changes observed in 1.5ppm water-accommodated fractions of crude oil exposure
  - Enlargement of gills and substantial increase in the number of mucus cells.
  - Mild hyperplasia and hypertrophy of the lamellae.
  - The epithelial cells of primary and secondary lamellae were swollen and oedematous and filled with red blood cells.

* Changes observed in 3.0ppm water-accommodated fractions of crude oil exposure
    - Extensive lamellar epithelial hyperplasia resulted in thickening and blunting and fusion of secondary lamellae.
    - Clubbing of secondary lamellae.
    - Sloughing off of secondary lamellae.
    - Lamellar telengiectasis (or aneurysm) of secondary lamellae.
    - Blood vessels were frequently packed with red blood cells (RBCs) especially near the gill filament.
* Changes observed in 5.0ppm water-accommodated fractions of crude oil exposure
    - Hyperplasia intensified towards the distal tip of the filament than near the base.
    - Thickening of the basement membrane.
    - Cells of secondary lamellae necrosed and rupture of lamellar tips causing haemorrhage.

### 7.1D. DISCUSSION

Cellular responses to pollutant-induced subleathal injury provided highly sensitive indicators of environmental impact (Hose *et al.*, 1996). Petroleum hydrocarbons have been reported to cause structural damage to the respiratory lamellae of the gills (Di Michele and Taylor, 1978; Poirier *et al.*, 1986; Correa and Garcia, 1990; Prasad, 1991). Crude oil and their

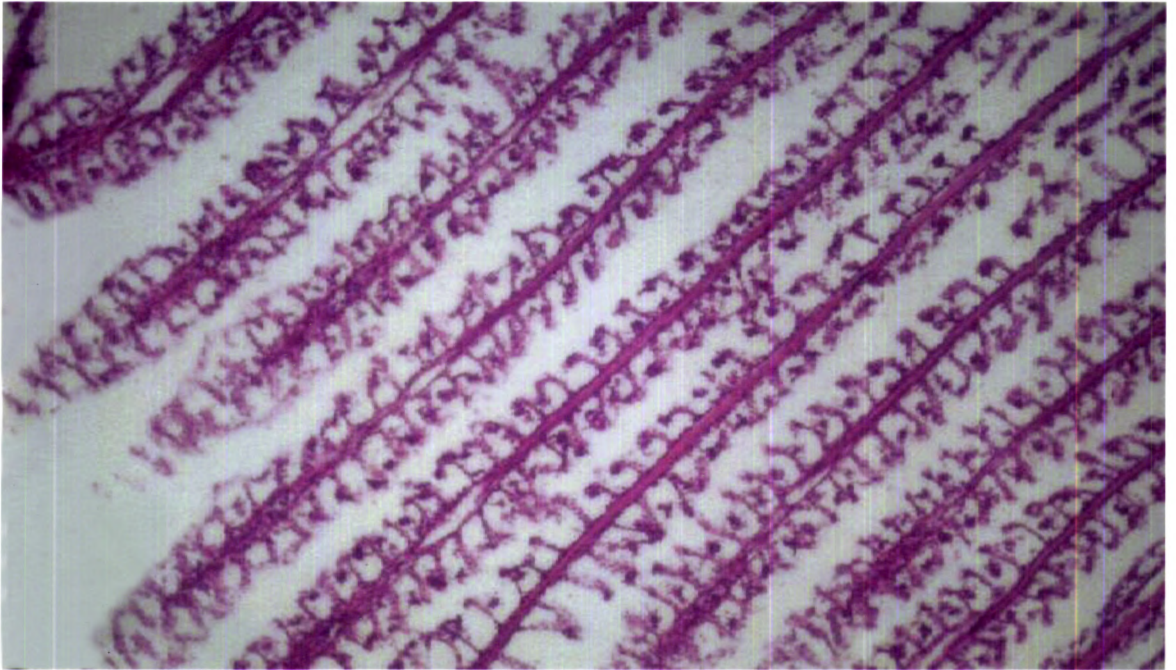


**PLATE-1** SECTION OF NORMAL GILL OF ***O.MOSSAMBICUS***

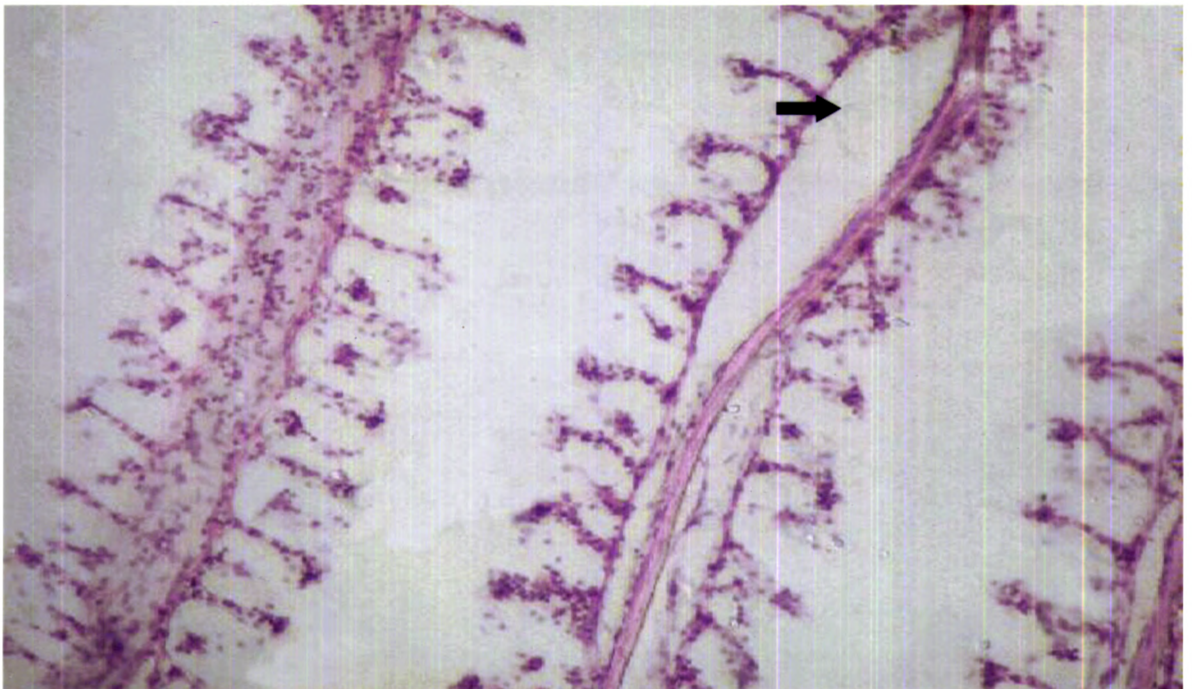


**PLATE-2** OEDEMA AND HYPERPLASIA IN PRIMARY AND SECONDARY LAMELLAE (40X)

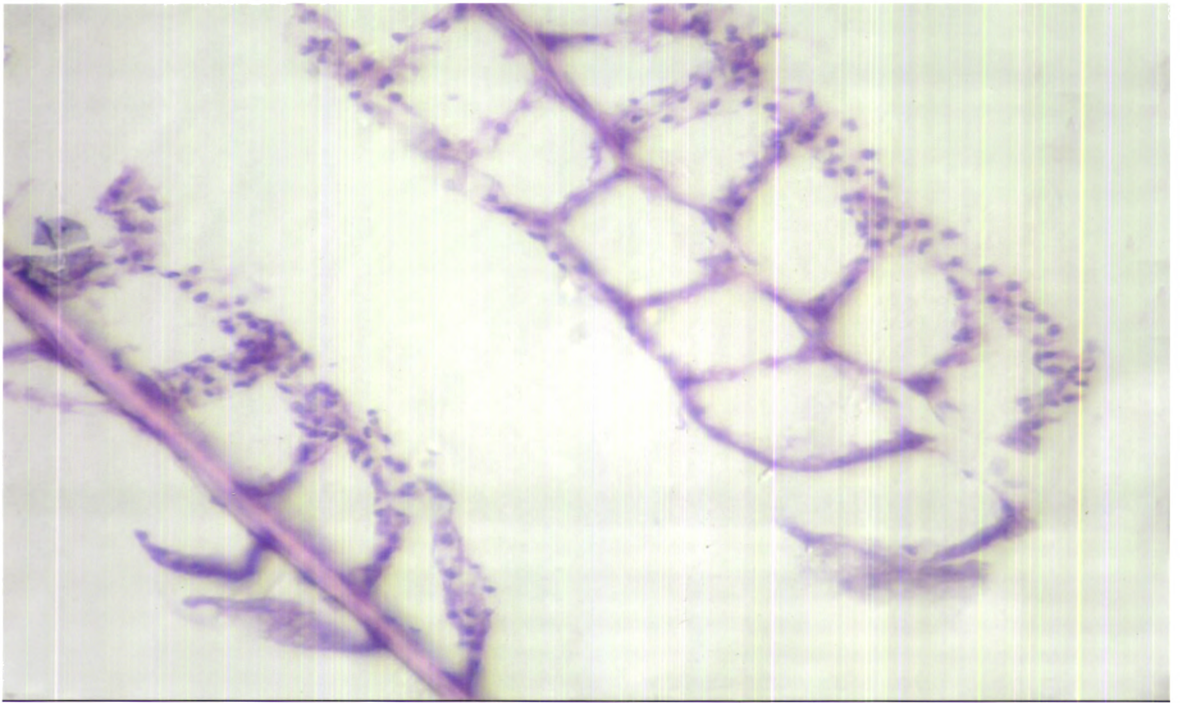


**PLATE-3** FUSION OF SECONDARY LAMELLAE (40X)

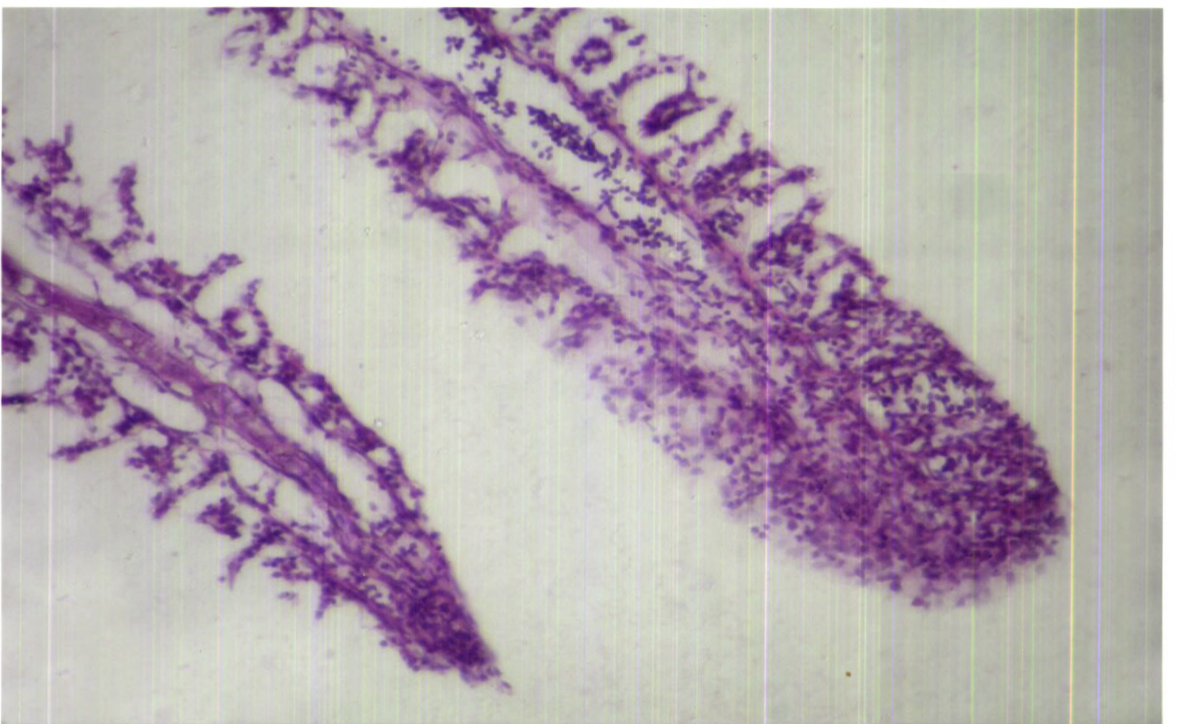


**PLATE-4** CLUBBING OF SECONDARY LAMELLAE (40X)

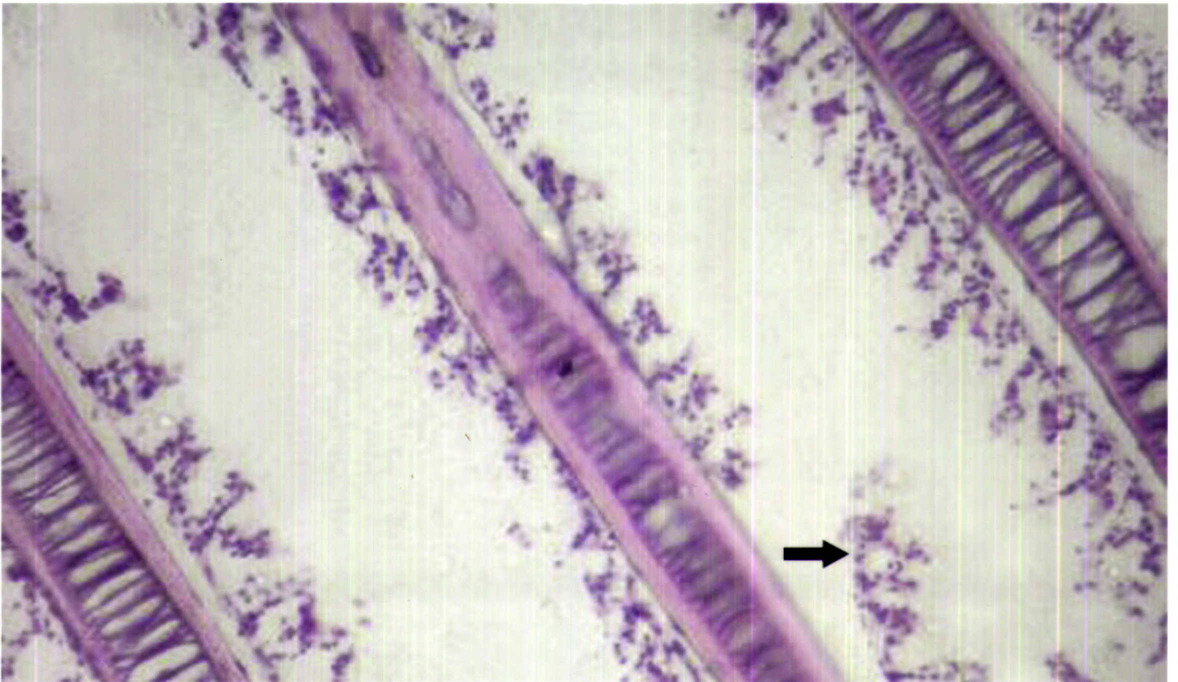


**PLATE-5** **SLOUGHING OFF OF SECONDARY LAMELLAE (40X)**

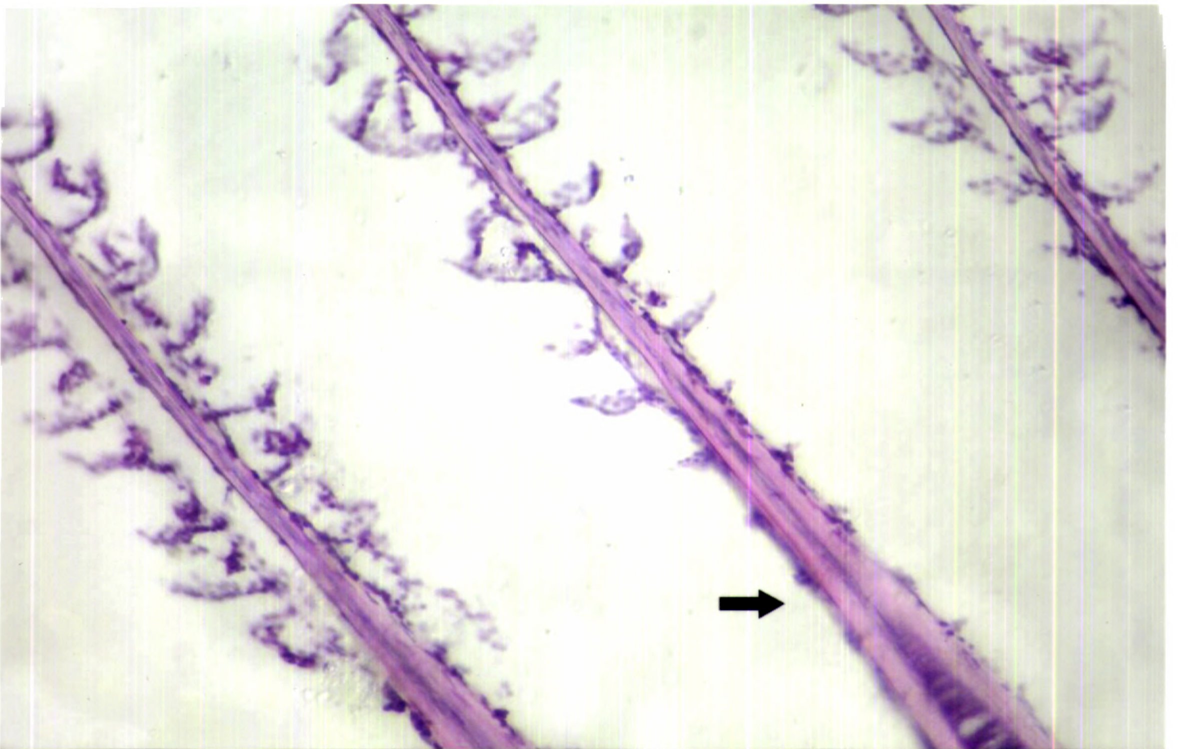


**PLATE-6** **NECROSIS OF GILL LAMELLAE (40X)**

fractions cause pathological changes in gills, liver, olfactory organ and other organs of fishes (Gardner, 1975). Damage to these vital organs eventually results in asphyxiation, reduced chemoreception, abnormal behavior or other conditions that can lead to death (Bue *et al.*, 1998).

In the present study when *O.mossambicus* was exposed to 1.5ppm, 3.0ppm and 5.0ppm of water-accommodated fractions of crude oil showed enlargement of the gills. The mode of feeding in *O.mossambicus* is such that the toxicant, mainly in dissolved form comes in contact with the gill filament first. On sensing the toxicant, the animal responds by secreting excess quantities of mucus thereby subjecting the concerned cells to become over-active and this probably may results in the enlargement of mucus cells rendering them less efficient. Therefore, though the secretion of excess quantities of mucus is a very useful defense mechanism, prolonged exposure to petroleum hydrocarbons would render the gills less efficient with reference to performance. The fact that the enlargement of gill cells is often accompanied by the presence of red blood cells indicates tissue damage as well, mainly in the form of inflammation. Prasad (1991) also revealed similar changes in *Anabus testudineus* after 48h exposure to crude oil.

Branchial oedema (Plate 2) was also observed in fish exposed to 1.5ppm water-accommodated fractions of crude oil along with excess of mucus secretion. Such effects would be predicted to impede gas exchange, and results in hypoxemia, which might be expected to stimulate catecholamine which alters its metabolic activities (Perry *et al.*, 1989; Randall

and Perry, 1992). Correa and Garcia (1990) have reported branchial oedema in the juvenile white mullet, *Mugil curema* under exposure to crude oil stress.

Lamellar telangiectasis (or aneurysm-localised dilation of lamellar sinusoids) of secondary lamellae and fusion of secondary lamellae (Plate 3) was observed in 3.0ppm dosed experimental animals and the result was in agreement with the report of Sandbacka *et al.*, (1999). Khan (1995) has reported lamellar epithelial hyperplasia and fusion of adjacent filaments in the gills of Atlantic cod, *Gadus morhua L.* exposed to water-soluble fractions of Venezuelan and Hiabernia crude oil at concentrations of 50-300ppb for 12 weeks. Exposure of cut throat trout for 90 days in the laboratory to concentrations as low as 50 µg/l of crude oil also resulted in hyperplasia of the gill lamellar filaments (Robert *et al.*, 1996). In the present study also lamellar hyperplasia was evident at all levels of exposure of water-accommodated fractions and hyperplasia was generally more pronounced towards the distal tip of the lamellar filament than near the base. Degrees of hyperplasia from slight to extensive occurred among filaments depending upon the concentrations of water-accommodated fractions of crude oil. Hyperplasia resulted in fusion of many secondary lamellae and also clubbing of secondary lamellae (Plate 4) in 3.0ppm exposed animals, which markedly reducing the respiratory surface area of some filaments. Mucus cells often occurred abundantly in the hyperplastic tissues. By the termination of the experiment, most lamellae were extensively hyperplastic and fused. The respiratory epithelium of many non-fused lamellae detached from underlying tissue. Kiceniuk *et al.* (1990) reported that exposing cod, *Gadus morhua*, to Venezulan crude oil at concentrations of 150-300ppb in water for 13 weeks,

produced fusion of gill filament and oedema in many of the secondary lamellae. Woodward *et al.* (1981) reported hyperplasia, oedema and fusion of gill lamellae of groups of *Salmo clarki* exposed to 450ppb and 520ppb of crude oil for 90 days.

Hawkes (1977) reported that 5 days of exposure of starry flounder, *Platichthys stellatus* to 100ppb of water-soluble fractions of crude oil caused sloughing off of gill epithelium. In the present study also, a sloughing off of gill epithelium (Plate 5) was noticed in the 3.0ppm dosed animals. Apart from this, at the same concentration, blood vessels were frequently packed with red blood cells (RBCs) especially near the base of the gill filament. Such type of hemorrhage was seen in *Menidia beryllina* (Cope) and *Trinectes maculates* (Block and Schneider) exposed to crude oil and its water-soluble fractions (Solangi *et al.*, 1982).

In the present study, fish exposed to the highest dose of water-accommodated fractions of crude oil namely 5.0ppm for 3 weeks revealed basement membrane thickening. This type of thickening of the basement membrane was usually associated with both inflammatory and hyperplastic lesions. The thickening membranes were characterized by discrete bands of homogeneous pale eosinophilic material bordering the branchial epithelium. Similar type of result was reported by Robert *et al.* (1996) in gill lamellae of rainbow surfperch, *Hypsurus caryi* exposed to petroleum seep.

Kohler (1990) and Khan (1995) have individually reported severe necrosis in the lamellae of flounder, *Platichthys flesus* and *Pleuronectes americanus* exposed to different types of crude oil. In the present study also

animals exposed to 5.0ppm of WAF showed severe necrosis of gill lamellae (Plate 6) and rupture of lamellar tips causing collapse of blood capillaries resulting in haemorrhage, which is evident by microcytic anaemia in our present study (Chapter 5). Whereas gill of control fish had normal filaments and lamellae. During the course of our study, it was evident that petroleum hydrocarbons acted as an agent to increase the fragility of erythrocyte membrane (Chapter 5), thereby spilling erythrocytes into the surrounding medium. This supports the collapse of the capillaries of the affected lamellae. Moreover, massive necrosis of the gill lamellae may be due to the labilisation of the lysosomal membrane induced by petroleum hydrocarbons leading to the leakage of lysosomal enzymes-β-glucuronidase and acid phosphatase to soluble fraction from ruptured lysosomes which is believed to enhanced cell damage and possibly cell death.

In summary, results from this study indicate that higher stress in gill tissue due to various concentrations of water-accommodated fractions of Bombay High crude oil is likely to deteriorate the structural integrity of the gills of *O.mossambicus*. Tissue alterations in this vital organ thus apparently induced hypoxia which leads to alterations in the metabolic processes of the fish.

## 7.2. INCIDENTAL PATHOLOGY

### 7.2A. INTRODUCTION

Pollution has been strongly linked with the outbreak of infectious disease of fish (Hillman, 1992; Anderson, 1993). Paul *et al.* (2000) has suggested that if the occurrence of stress coincides with the presence of micro organisms, it is logical to assume that outbreaks of diseases are more likely to take place. Considering the complex nature of the aquatic environment coupled with the ubiquitous nature of the potential bacterial fish pathogens, it is possible that the stresses caused by a pollutant like petroleum hydrocarbons can trigger epizootics. Opportunistic pathogens amongst the normal bacterial flora of aquatic animals can cause infections when the animal is stressed.

Disease resistance and pathology induced by petroleum hydrocarbons have been well documented with a majority of workers concentrating on the effects on fish. Crude oil, which is spilled into the oceans continually is a toxicant known to impair the health of fish, but its effects on fish parasites are more subtle-some may be favoured but others not. Oil pollution can also act at sublethal concentrations by impairing the fish's defense mechanisms against common fish parasites (Khan, 1987). Paul *et al.* (2000) observed that when juveniles and sub adults of the penaeid shrimp *Metapenaeous dobsoni* (Miers) exposed to lethal and sublethal concentrations of water-accommodated fractions of Bombay High crude oil developed symptoms of vibriosis. Marty *et al.* (1999) observed dose-dependent expression of viral haemorrhagic septicaemia virus (VHSV) in

adult Pacific herring *(Clupea pallasi)* following Exxon Valdez oil spill in Prince William Sound. Minchew and Yarbrough (1977) observed that mullet *(Mugil cephalus)* when exposed to low levels of crude oil in four estuarine ponds developed fin rot and the suspected pathogen was identified as a species of the genus *Vibrio*.

A variety of micro-organisms cause diseases in marine animals exposed to toxicants (Mix, 1988). Among pathogenic bacteria, motile *aeromonads* of the *Aeromonas hydrophila* species cause tail/fin rot and haemorrhagic septicaemia in fish (Bullock *et al.*, 1971; Schaperclaus *et al.*, 1992). Hazan *et al.* (1979) reported that *Aeromonas* are ubiquitous, oxidase positive, facultatively anaerobic, glucose fermenting gram negative bacteria that are native to aquatic environments. The pathognomic signs of motile aeromonad haemorrhagic septicaemia are closely linked to the capacity of the relevant species to induce rupture of minor blood vessels. The haemorrhages caused by a soluble haemolysin, which has been cloned and characterized (Aoki *et al.*,1983) may be associated with ulcerative skin lesions (which lead to the sloughing off of the scales), and may be on the surface of organs or deep within tissues, local haemorrhages particularly in the gills and vent, ulcers, abscesses, exophthalmia and abdominal distension. External lesions may vary from an extensive superficial reddening of the surface of a large area of the body, often with necrosis of fins or tail (fin rot), to extensive ulceration over a considerable portion of the flanks or dorsum (Valerie *et al.*, 1993). Internally, there may be accumulation of ascitic fluid, anaemia and damage to the organs, notably liver, gill and kidney (Huizina *et al* 1979) This conditions which may reach epizootic proportion,

is characterized by erosion of the scales and pin-prick haemorrhages which may cover up to 75% of the body surface (Carls *et al.*, 1998).

*Aeromonas* species are important members of the normal micro flora and recently there has been an increasing appreciation of their role as water borne pathogens of fish and man (Pathak *et al.*, 1988). *Aeromonas hydrophila* is found to be significantly associated with fish and shell fish diseases (Popoff, 1984) and it is typically recognized as an opportunistic pathogen or secondary invader (Austin and Austin, 1987).

Walters and Plumb (1980) while studying the influence of stress factors on the virulence of *A. hydrophila* in cat fish found that low oxygen (1.5 mg/l) and high ammonia (1.2 mg/l) significantly influenced the percentage of mortality Mac Millan and Santucci (1990) have reported that occurrence of infectious diseases in cultured channel cat fish caused by *A.hydrophila* under the influence of high levels of carbondioxide, ammonia, nitrite and low oxygen. Occurrence of haemorrhagic septicaemia in Indian major carps caused by *Aeromonas* in tanks loaded with high levels of organic manure were studied by (Son *et al.* (1997).

The present study focuses on the occurrence of disease induced by the stress of petroleum hydrocarbons in *Oreochromis mossambicus* and identification of the aetiological agent.

### 7.2B. MATERIALS AND METHODS

Koch's Postulates were tested in four steps to determine the aetiological agent of the disease.

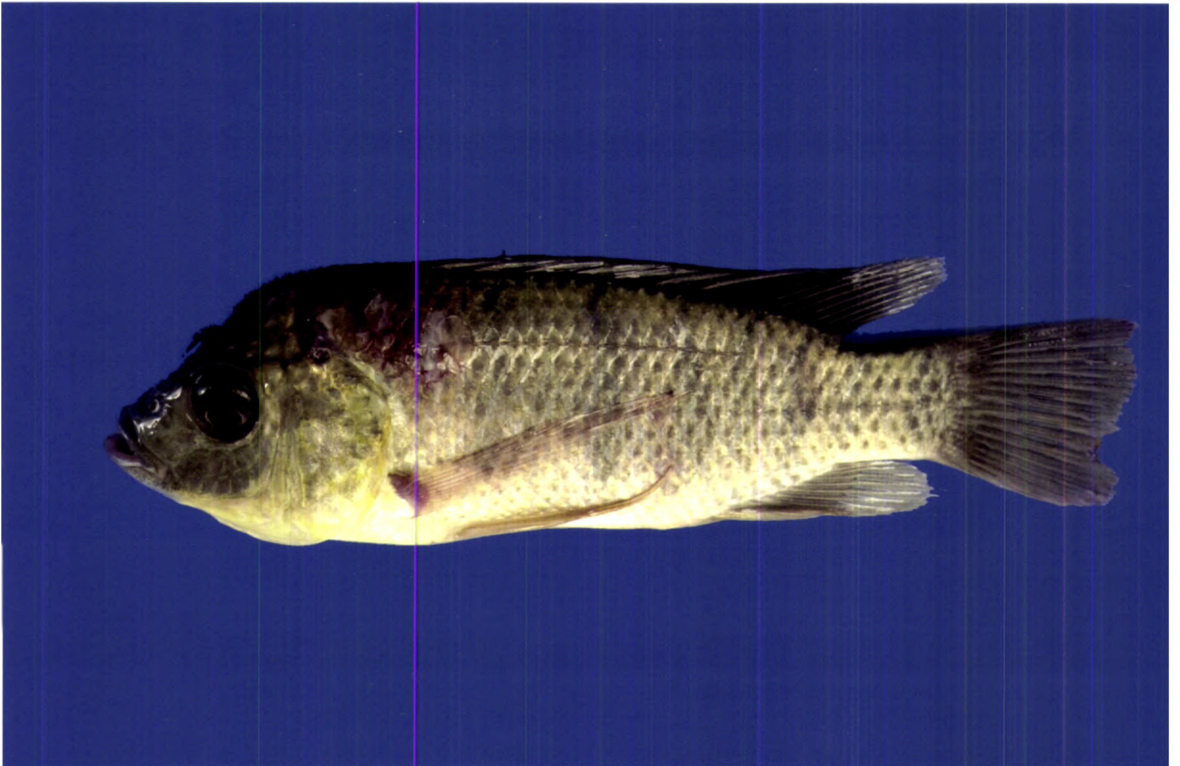


**PLATE-7** **HAEMORRHAGIC SEPTICAEMIA IN *O.MOSSABICUS***

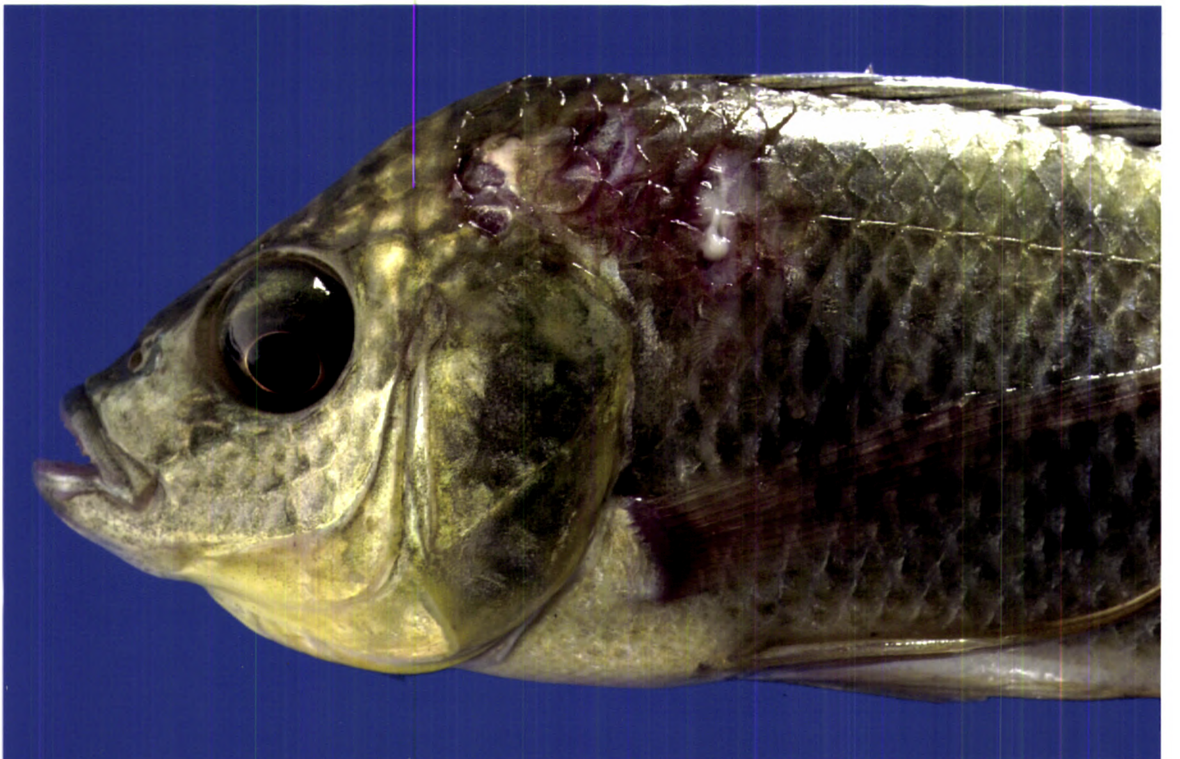


**PLATE-8** **HAEMORRHAGIC SEPTICAEMIA IN *O.MOSSABICUS***

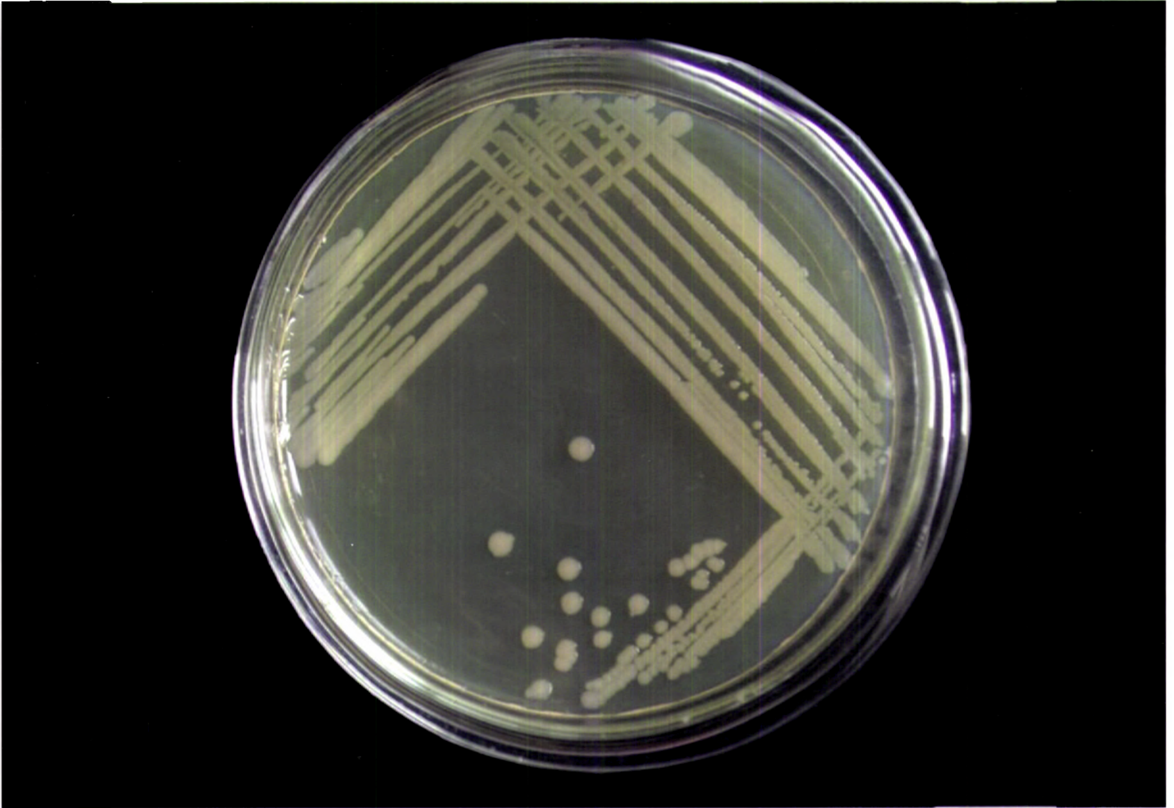


**PLATE-9** ***AEROMONAS HYDROPHILIA*** **COLONIES ON TSA MEDIUM**

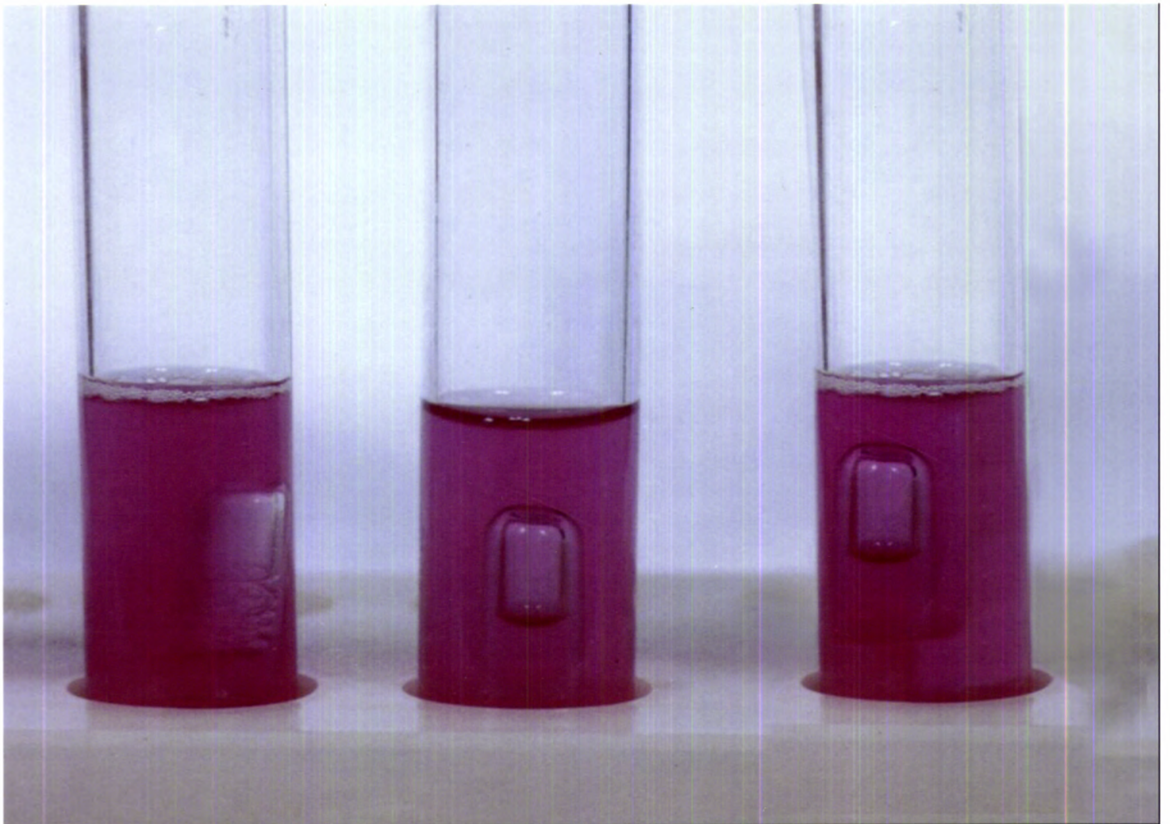
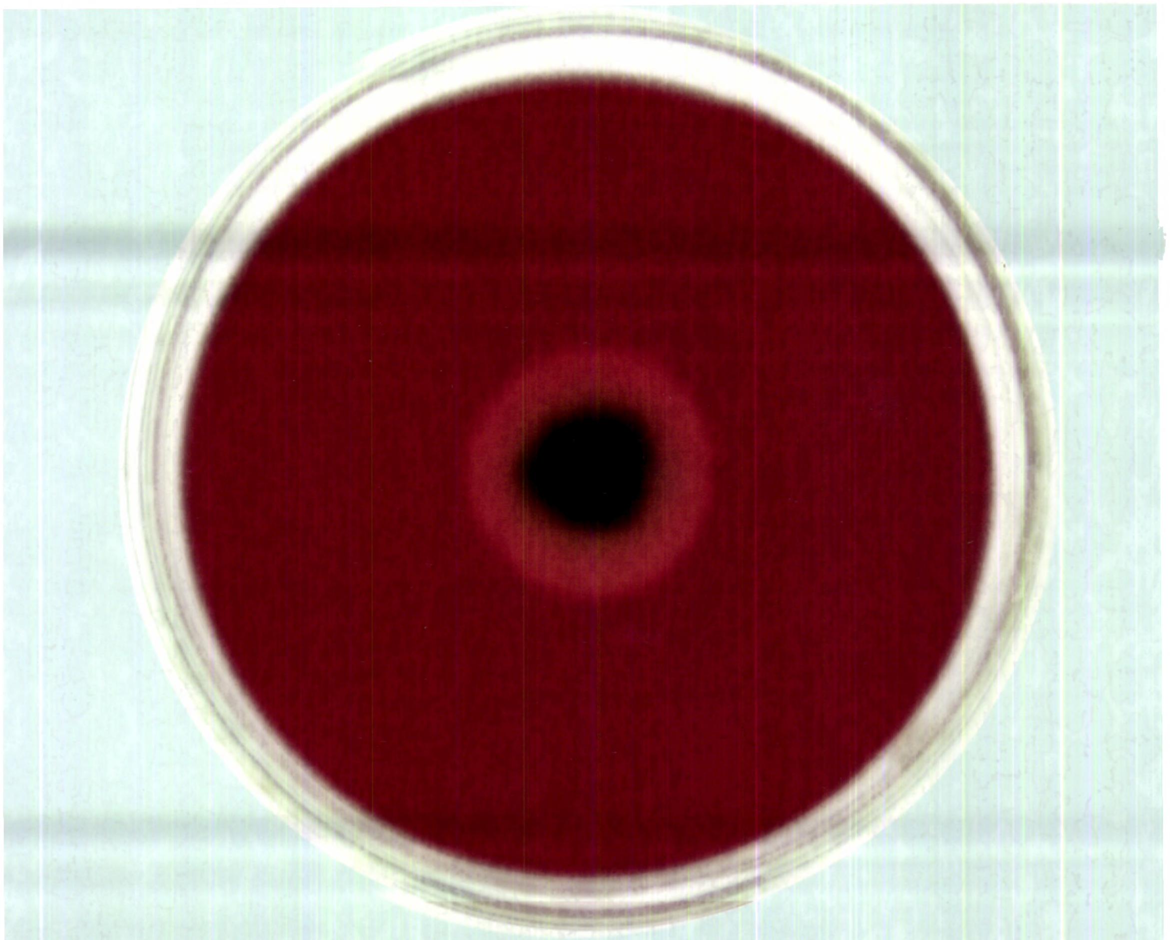


**PLATE-10 FERMENTATION OF GLUCOSE BY** ***AEROMONAS HYDROPHILIA***

**PLATE-11** HAEMOLYTIC ACTIVITY OF *A.HYDROPHILA* ON BLOOD AGAR



### 7.2B.1. Determination of pathogen through Koch's postulates

#### a. Isolation of pathogen

The rearing water, blood and muscle tissue with lesion of the diseased fish were the samples used for the isolation of the pathogen. Tripticase soya agar (TSA) (Tryptone- 15 g; Soyapeptone-5g; Sodium chloride- 5g; agar-20g; distilled water- 1000ml, pH 7.3) was used for the study. Spread plate method for water, streak inoculation for blood sample and impression smear for muscle tissue was employed. The plates were incubated at 28 ± 2°C in a Bacteriological incubator for 3 days. Colonies were observed and isolated into TSA slants. These cultures were repeatedly streaked for purification and the pure colonies were stocked in TSA vials overlaid with sterile liquid paraffin.

#### b. Identification of pathogen

Standard methods for identification of the pathogen (Baumann *et al.*, 1984) such as Gram staining, motility test, marine oxidation-fermentation test, oxidase test, production of hydrogen sulphide, utilization of arabinose, arginine, histidine, Voges-Proskauer test, vibriostat sensitivity test, gas production from glucose, salicin fermentation test, aesculin degradation test and blood haemolysis were performed and the pathogen was identified upto generic level.

#### c. Reinfecting the healthy host (*O.mossambicus*) with the pure isolate

Suspension of the isolate in sterile saline was prepared by harvesting an eighteen-hour-old slant culture with physiological saline. Absorbance of the suspension was measured at 600nm in a Hitachi model 200-20 UV-Visible Spectrophotometer and the corresponding cell number

was estimated by direct counting of the Gram stained smear preparation using a Nikon Optiphot-2 Research Microscope.

*O.mossambicus* of size 15 ± 3g were maintained separately in 5.0ppm, 3.0ppm and 1.5ppm concentrations of water-accommodated fractions of crude oil for 5 days. A control set was maintained without the addition of water-accommodated fractions of crude oil. Dilutions containing the pathogen at concentrations of $1 \times 10^4$ cells to $1 \times 10^6$ cells per ml were prepared using sterile saline and injected subcutaneously into the experimental animals as well as to the control set. The manifestation of disease and mortality of the experimental animals were monitored.

### d. Reisolation of the pathogen

Isolation of the pathogen from diseased fishes was performed as given in 7.2 B.1.b. The pathogen isolated was again identified using the standard biochemical tests mentioned above.

## 7.2C. RESULTS

### Bacteria associated with haemorrhagic septicaemia in *O. mossambicus*

**Table 7.2.1 Presence of *Aeromonas hydrophila* in rearing water, blood and muscle during the culture period**

| Culture Period (days) | Sample | Presence of *Aeromonas hydrophila* |
|---|---|---|
| 0 | Rearing water | + |
| | Blood | |
| 21 | Rearing water | + |
| | Blood | + |
| | Muscle tissue | + |

**Table 7.2.2 Morphological and Biochemical tests for the identification of the pathogen**

| Sl. No. | Biochemical Characteristics | Response |
|---|---|---|
| 1 | Gram Staining | |
| 2 | Mannitol Motility | + |
| 3 | Production of Hydrogen sulphide gas | + |
| 4 | Oxidase test | + |
| 5 | Utilisation of arabinose | + |
| 6 | Utilisation of arginine | + |
| 7 | Utilisation of histidine | + |
| 8 | Voges-Proskauer reaction | + |
| 9 | Marine oxidation fermentation | $\mp^{o}$ |
| 10 | Vibriostat sensitivity | |
| 11 | Gas production from glucose | + |
| 12 | Salicin fermentation | + |
| 13 | Degradation of aesculin | + |
| 14 | Blood haemolytic activity | + |

**Table 7.2.3 Responses of *O.mossambicus* challenged with *A.hydrophila***

| Response | Treatment (WAF) | *A.hydrophila* (Cells/animal) | | |
|---|---|---|---|---|
| | | $10^6$ cells/animal | $10^5$ cells/animal | $10^4$ cells/animal |
| Mortality | Control | 0 | 0 | 0 |
| | 1.5ppm | 0 | 0 | 0 |
| | 3.0ppm | 33 | 17 | 0 |
| | 5.0ppm | 33 | 33 | 17 |
| Disease manifestation | Control | 0 | 0 | 0 |
| | 1.5ppm | 0 | 0 | 0 |
| | 3.0ppm | 50 | 33 | 0 |
| | 5.0ppm | 67 | 50 | 33 |

⇒ Responses are expressed in percentage n = 6

The TSA plates inoculated with blood and muscle tissue (Table 7.2.1) of the diseased fish (Plate 7 and 8) was found to be dominated with a single type of cream coloured colony (Plate 9). These colonies were isolated and tested for morphological and biochemical characteristics. The isolates were Gram-negative short rods, fermentative (Plate 10), oxidase positive due to monotrochous polar flagellum and have the ability to produce hydrogen sulphide gas from cysteine and utilize histidine, arginine and arabinose. They also showed positive result for the Voges-Proskaeur test and have the property to degrade aesculin. All the strains were resistant to vibriostat compound and highly haemolytic in nature (Plate 11). Based on these biochemical characteristics the pathogen was identified as *Aeromonas hydrophila* (Table 7.2.2).The rearing water was found to harbour *Aeromonas hydrophila*. *Aeromonas hydrophila* isolated from blood and muscle tissue of the diseased fish shows the clinical signs of bacterial haemorrhagic septicaemia.

When the animals were reinfected with the isolate (Table 7.2.3), the maximum disease manifestation (67% for $10^6$ cells/animal; 50% for $10^5$ cells/animal and 33% of $10^4$ cells/animal) and mortality (33% for $10^6$ cells/animals and $10^5$ cells/animal and 17% of $10^4$ cells/animal) could be observed in fish exposed to 5.0ppm water-accommodated fractions of crude oil. Moderate disease manifestation (50% for $10^6$ cells/animal; 33% for $10^5$ cells/animal and 0% of $10^4$ cells/animal) and mortality (33% for $10^6$ cells/animal; 17% for $10^5$ cells/animal and 0% of $10^4$ cells/animal) could be noticed in 3.0ppm dosed experimental animals. No indication of disease and mortality was seen in 1.5ppm dosed experimental animals. This may be due to

immunostimulation by the low doses (1.5ppm) of water-accommodated fractions of crude oil. At 5.0ppm, disease manifestation and mortality could be observed with all the doses ($10^4$ $10^5$ and $10^6$ cells/animal) of the pathogen. However, lower doses of the pathogen ($10^4$ cells/animal) neither induce disease nor mortality in 3.0ppm dosed experimental animals. Therefore the optimal dose for reproducing the disease is found to be $10^5$ cells/animal. In the control group there was no disease manifestation and no mortality.

## 7.2D. DISCUSSION

In the present study the presence of petroleum hydrocarbons was found to induce haemorrhagic septicaemia in *O.mossambicus.* The aetiological agent of this haemorrhagic septicaemia is *Aeromonas hydrophila* which is a natural flora of fresh water which acts as an opportunistic pathogen. The water-accommodated fractions of crude oil may cause stress on the experimental animal and weaken the defense system of the animal. This may promote the invasion of the pathogen *(A.hydrophila)* into the susceptible host *(O.mossambicus)* resulting in haemorrhagic septicaemia. The decrease in WBC count already established in the previous chapter (Chapter 5.2) suggests that WAF exposed fish have decreased capacity to defend against microbial or bacterial infection. Schotts *et al.* (1972) have stated that *aeromonads* are autochthonous inhabitants of aquatic environments and colonise on skin surface and intestinal lumen of fishes. It was reported as an opportunistic pathogen or secondary invader by Austin and Austin, 1987 Similar incidence of stress induced occurrence of haemorrhagic septicaemia has been reported in major carps by Son *et al.*

(1983). Paul *et al.* (2000) noticed the occurrence of vibriosis in *M.dobsoni* exposed to crude oil. These observations strongly suggest the role of pollutants in the outbreak of infectious disease in fish. When environmental stress coincides with the presence of the pathogen, a susceptible host may succumb to the disease.

*A.hydrophila* is a natural flora present in fresh water. In the present study also it was found to be present in the rearing water. For testing Koch's Postulates, *A.hydrophila* was injected into *O.mossambicus* exposed to water-accommodated fractions (1.5ppm, 3.0ppm and 5.0ppm concentrations) as well as control. Disease manifestation and mortality could be observed only in fishes exposed to 3.0ppm and 5.0ppm concentrations of water-accommodated fractions. However fishes exposed to 1.5ppm of water-accommodated fractions and the control group was seen to be unaffected. This shows the opportunistic nature of the pathogen requiring a stress factor for invasion into a susceptible host whose immune system had been weakened by the pollutants in the system.

The motile *aeromonad* infections can at times cause devastating losses in both wild and farmed fish population. Since they are almost invariably associated with the invasion of a pathogenic strain into a stressed population, control, at least in farmed populations, has to be aimed primarily at reducing the level of possible stressors.

*Chapter* 8

# Summary And Conclusions

The present study investigated the biochemical and histopathological impacts of different concentrations (1 5ppm, 3.0ppm and 5.0ppm) of water-accommodated fractions of Bombay High crude oil on a freshwater- adapted euryhaline teleost *O.mossambicus* for three weeks. Summary of the important findings are:

- The surfacing movements exhibited by the fish under exposure to water-accommodated fractions (WAF) of crude oil may be a testimony of respiratory stress.
- Depletion of total carbohydrates and glycogen which form the major energy currency, with the simultaneous increase in blood glucose levels suggesting breakdown of carbohydrates to meet energy crisis during petroleum hydrocarbons toxicity
- The decrement in pyruvate level with a concomitant increase in lactate dehydrogenase and lactic acid suggests a tendency of a shift towards anaerobic dependence than aerobic oxidation.
- Increased phosphorylase 'a' activity with the significant decrease in phosphorylase 'b' activity in WAF exposed fish suggests increased breakdown of glycogen to meet higher energy demands.
- Increased activities of Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in all tissues indicate enhanced oxidation of glucose through HMP pathway during WAF toxicity.

* Significant inhibition of succinate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase in WAF exposed fish indicates impaired oxidation of carbohydrates through TCA cycle.

* Decreased cytochrome-c-oxidase activity in the tissues of WAF exposed fish demonstrating decrement in the mitochondrial ability towards ATP synthesis.

* Significant reduction in protein content with the corresponding increase in free amino acids and protease activity suggests an increased breakdown of protein under petroleum hydrocarbons stress.

* Increased levels of ammonia and urea in WAF intoxicated fish indicates an increased catabolism of protein and also other nitrogenous compounds.

* Glutamate dehydrogenase activity is significantly inhibited in all tissues under WAF exposure suggesting accumulation of glutamate which may aid in meeting the energy demands under petroleum hydrocarbons stress by being fed into the TCA cycle through increased activities of aspartate amino transferase and alanine amino transferase.

* The antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase as well as glutathione were significantly increased in 1.5ppm and 3.0pm WAF exposure.

* On exposure to 5.0ppm concentration of WAF, the levels of conjugated dienes, hydroperoxide and malondialdehyde were significantly increased

with the corresponding decrease in antioxidant enzymes as well as antioxidant-glutathione suggesting that a severe oxidative stress was experienced by fish exposed to higher concentrations of petroleum hydrocarbons.

* Exposure to different concentrations of WAF damages the lysosomal system involving the loss of integrity of the lysosomal membrane, thereby resulting in 'leaky' lysosomes which can significantly release its hydrolytic marker enzymes such as β-glucuronidase and acid phosphatase to the cytosol.

* Exposure to petroleum hydrocarbons caused labilisation of erythrocyte membrane.

* Significant decrement in WBC, RBC counts as well as haemoglobin level with the corresponding increase in haematocrit value indicate that fishes intoxicated with petroleum hydrocarbons are highly susceptible to infection, demonstrating the lack of resistance.

* Branchial membrane-bound enzymes like $Na^+$-$K^+$ATPase and $Mg^{2+}$ATPase were significantly inhibited by all concentrations of WAF exposure.

* Significant accumulation of petroleum hydrocarbons could be detected in liver, gill and muscle tissues.

* Order of toxicity of WAF for *O.mossambicus* is. 5.0ppm > 3.0ppm > 1.5ppm.

* Exposure to different concentrations of WAF resulted in varying degrees of damage to gill tissue. The histopathological alterations observed in gill tissue are: oedema, hyperplasia, fusion of secondary lamellae, sloughing off of secondary lamellae, telengiectasis, clubbing of secondary lamellae, thickening of the basement membrane and finally severe necrosis.

* Petroleum hydrocarbons weaken the defense system of the fish and promote the invasion by the opportunistic pathogen- *Aeromonas hydrophila* resulting in haemorrhagic septicaemia.

The overall result of this study revealed considerable changes in enzyme activities and other parameters representing disturbances in metabolism of *O.mossambicus,* either to encounter the increased energy demand during the stress caused by petroleum hydrocarbons or as possible steps towards detoxifying processes.

In this perspective backdrop, it can be concluded that exposure of *O.mossambicus* to petroleum hydrocarbons for three weeks is highly stressful and different parameters in this study can be adapted for future investigations as biomarkers of damage caused by petroleum hydrocarbons to aquatic organisms.

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