HAEMATOLOGICAL AND TOXICOLOGICAL STUDIES ON BRACKISH WATER FISH *ETROPLUS MACULATUS* (BLOCH)

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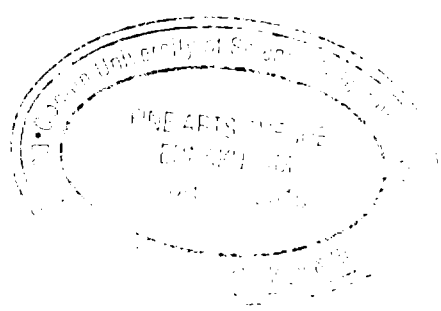

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This is to certify that the thesis entitled "**Haematological and Toxicological studies on brackish water fish *Etroplus maculatus* (Bloch)**" is an authentic record of the research work carried out by **Mrs. Bindu Bhaskaran. A.B** under my guidance and supervision in the department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Marine Biology of Cochin University of Science and Technology and no part of these has been presented for the award of any other degree, diploma or associate ship in any university.

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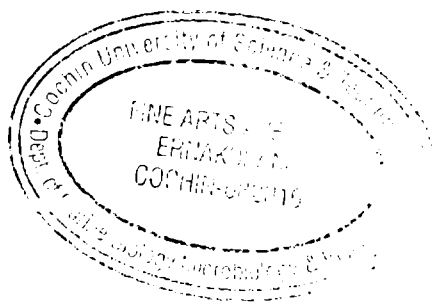

DECLARATION

I hereby declare that the thesis entitled "**HAEMATOLOGICAL AND TOXICOLOGICAL STUDIES ON BRACKISH WATER FISH *ETROPLUS MACULATUS* (BLOCH)**" is a genuine record of research work done by me (Reg. No. 2148) under the guidance and supervision of Prof. (Rtd) Dr. K.Y.Mohammed Salih, Department of Marine Biology, Microbiology and Biochemistry, in partial fulfillment of the degree of Doctor of Philosophy under the faculty of Marine Sciences of the Cochin University of Science and Technology and no part thereof has been previously formed the basis of the award of any degree, diploma or associateship in any university.

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Chapter 1
GENERAL INTRODUCTION

INTRODUCTION

1.1 HAEMATOLOGICALSTUDIES

Among the various animal groups, fishes have been identified as being very sensitive to pollutants and have been the most popular test organism because they are presumed to be the best understood organism in the aquatic environment. Many of the clinical tools used to evaluate mammalian health are not developed for use in fishes. As the aquaculture industry expands, there is an increasing need for improved diagnostic methods. There are few tools available to diagnose and monitor diseases on fishes. One such tool is the analysis of blood; it can help detect acute and chronic pathophysiological changes attributable to nutrition, water quality, toxicants and disease.

One of the difficulties in assessing the state of health of natural fish population has been the paucity of reliable references of the normal condition. In pursuit to this goal, many fish physiologists have turned to studies of haematology. Haematological data are used routinely in health care of humans and domestic animals. Haematological evaluations are also gradually becoming a routine practice for determining health status in fish (Tavares-Dias *et al.*, 2003; Ranzaini-Paiva *et al.*, 2003; Cazenave *et al.*, 2005). Only a few normal values for small number haematological parameters have been established for some teleosts, but these values range widely due to the lack of standardized collecting and measuring techniques (Blaxhall, 1972). Perhaps further confounding these data are variables such as age, sex, dietary state, and stress, all of which may alter blood values (Barnhart, 1969; McCarthy *et al.*, 1973).

As the aquaculture industry expands, tools to monitor the health status of fish using standardized non-lethal and expressive methods will be needed (Hrubec *et al.* 2000). The use of haematological indices in commercial fish cultivation may

enhance production by facilitating early detection of situations of stress and/or diseases that could affect production performance (Tavares –Dias and Moraes, 2004; Rehulka *et al.* 2004, Tavares-Dias and Barcellos, 2005). This, in turn, will contribute to more specific, timely and effective diseases treatments in the future. The study of piscine haematology has contributed significantly to our understanding of the comparative physiology, phylogenetic relationships, habit, habitat, food selection, and other significant ecological parameters of fishes (Ayotunde and Ochang, 2004, 2006).

For the successful management of any type of hatchery and fish farm, knowledge of the species concerned is prerequisite. Unfortunately in most facilities, the stocking density is so high that stress syndromes and diseases are quite frequent. The sudden outbreaks of unknown etiology have caused not a little amount of set back in the aquaculture contingent. So along with the information about the food habits and habitat, the culturists should have the expertise to recognize the unhealthy from the healthy. "Since a change or lack of change in the blood picture is a fundamental; characteristic of practically every physiologic or pathologic state. Hematological finding are among the most valuable and most generally useful of all laboratory diagnostic aids" (Wells, 1956). Pickering(1986) suggested that the simplicity of most blood sampling techniques probably accounts for the wide spread use of blood studies as a means of assessing the state of health of teleost fish.

One of the principal functions of the blood is respiratory, and involves transporting oxygen from the gills to the tissues, and transporting carbon dioxide from the tissues to the gills. In addition, the blood also serves as a vehicle in absorption and transporting nutrients, vitamins and hormones, as well as removing waste products and protecting the body against certain infectious agents. From the point of view of the fish health specialist, one of the great practical advantages of the blood is that small samples can be taken for analysis without necessarily having to kill the animal itself.

Haematological parameters are often used as health status and stress indicators in fish as in mammals (Duthie and Tork, 1985); Gallardo *et al.* 2003;

Ballarin *et al.*, 2004). Haematological parameters are recognized as secondary stress indicators (Wedemeyer *et al.*, 1990; Wojtaszek *et al.*, 2002; Pierson *et al.*, 2004).

The blood parameters that are most frequently evaluated during routine haematological examinations of fish populations include the haemoglobin concentration, the haematocrit level, the red blood cell count, aspects of the morphology, distribution of the formed cellular elements and leucocytes in the blood. The haematological indices are used to assess the functional status and oxygen carrying capacity of the blood stream (Shah and Attendag, 2005). The haemoglobin content and erythrocyte count are directly related to environmental factors such as temperature and salinity (Graham, 1997), while immune system parameters are used to assess any alterations in the defense mechanism of fish (Jones, 2001). As such, an analysis of the normal haematological profile of a fish enables an expert to identify external and internal stress conditions and to predict the chances of disease.

1.2 AQUATIC POLLUTION

Pollution of water resources available on the earth, which has caused significant effects on the aquatic ecosystem (Trivedi and Goel, 1986) plants and animal life.

Increased demand for food and the need to sustain the ever increasing world population have led to massive increase in both agricultural and industrial activities. Today, India is one of the first ten industrialized countries of the world. India, like any other developing country, is faced with problems arising from the negative impact of economic development due to industrial pollution. With the advent of agricultural and industrial revolution, most of the water sources are becoming contaminated (Khare and Singh, 2002). Rapid progress made in industrialization without adequate environmental safety results in lack of good quality of water both for irrigation and drinking. Industrial effluents have been regarded as sources of pollution because of the lack of efficient treatment and disposal (Srivastava and Pandey, 1999).

The contamination of fresh waters with a wide range of pollutants has become a matter of concern over the last few decades (Dirilgen, 2001; Vutukuru, 2005). The contamination of fresh water systems thereby causing adverse effects on aquatic biota and human health (Wang, 2002; Dautremepuits *et al.*, 2004). The release of waste waters into the water bodies affects the flora and fauna (Nampoothery and Sasidhadaran, 1976; Singh *et al.*, 1976). Aquaculture activities are mostly confined to the coastal or inland water bodies, which are comparatively more polluted than the open ocean waters owing to their close proximity to human inhabitation. The rivers which are the lifelines of our culture and economy are dying because of severe pollution. A study conducted by National Environmental Engineering Research Institute (NEERI) shows that nearly 70 % of the river water in India is polluted (Martin, 1998).

River Periyar has been performing a pivotal role in shaping the economic prospects of Kerala, as it helps in power generation, domestic water supply, irrigation, tourism, industrial production, collection of various inorganic resources and fisheries. However, as in the case of many other inland water bodies, River Periyar is gradually undergoing eco-degradation throughout its course of flow due to various anthropogenic stresses, which include indiscriminate deforestation, domestic–agricultural–industrial water pollution, excessive exploitation of resources, large scale sand mining, various interferences in the flow of water etc.

Greenpeace study established that with most of the fertilizer, insecticide and chemical manufacturing plants in the Eloor- Edayar region dumping toxic waste into the Periyar, the incidence of almost all diseases, whether respiratory, dermatological or mental, was "two to five times higher in the region" compared to the less-polluted Pindimana village in the same district. Due to the presence of waste, the temperature of the river had risen abnormally and the river water contained high concentrations of organochlorides including DDT and its metabolites, endosulfan, cyanide, BHC and heavy metals including mercury, lead, cadmium, chromium and zinc (India Together, 2010).

Agricultural, industrial and domestic effluents generally contain a wide variety of organic and inorganic pollutants, such as solvents, oils, heavy metals,

pesticides, fertilizers and suspended solids (Gbem *et al.*, 2001; Woodling *et al.*, 2001; Pandey *et al.*, 2003). The alterations of the ecosphere by human activities may be physical, chemical, biological or radioactive. Chemical alteration of the environment appears to be the major type which threatens the living system extensively. Such contaminants change water quality and may cause many problems to fish, such as diseases and structural alterations (Chang *et al.*, 1998). All organisms maintain their "internal milieu" more or less constant by making use of a variety of regulatory mechanisms. When the level of pollutants in the environment exceeds the assimilatory capacity of these regulatory mechanisms, it leads to biochemical changes finally resulting in death.

The heavy metal and pesticide contamination of aquatic system has attracted the attention of researchers all over the world (Dutta and Dalal, 2008) owing to the toxicity of heavy metal at very low levels, persistence in the environment and ability to get incorporated in the tissues of organisms. All this factors make these toxicants deleterious to the aquatic environment and consequently to humans who depend on aquatic products as source of food. Heavy metals can accumulate in the tissues of aquatic animals and such as tissue concentrations of heavy metals can be of public health concern to both animals and humans (Kalay *et al.*, 1999; Ashraf, 2005).

1.3 HEAVY METAL POLLUTION

Heavy metals are a group of metallic elements with atomic weights greater then 40. In aquatic systems the heavy metals of greatest concern are copper, zinc, cadmium, mercury and lead. The term 'trace metals' identifies a large group of metallic elements which are present in living organisms in limited amounts that is which is required in amounts smaller than 0.01 % of the mass of the organism. Trace metals are usually divided into two sub-classes. The first includes Fe, Mn, Cu, Co, Mg and Zn which are essential micronutrients. Such nutrient metals are usually key elements in metallo enzymes or cofactors in enzymatic reactions. However, the micronutrients when present above threshold levels become toxic. Non essential trace metals, Cd, Hg, Cr, Pb etc. belong to the second category which

is made up of metals without any established biological function and include the more important contaminants in the aquatic environment.

Pollution of aquatic environments with heavy metals has seriously increased worldwide and under certain environmental conditions fish may concentrate large amounts of some metals from the water in their tissues (Mansour and Sidky, 2002). Bioaccumulation of nonessential metals in tissues leads to intoxication, decreased fertility, tissue damage and dysfunction of a variety of organs (Oliveira Ribeiro *et al.*, 2000; Damek-Proprawa *et al.*, 2003). It was reported that metals are taken up through different organs of the fish and induced morphological, histological and biochemical alterations, in the tissues which may critically influence fish quality (Olojo *et al.*, 2005; Fadel and Gaber, 2007). Heavy metals cause adverse biological effects (Lawrence, 1981; Zelikoff *et al.*, 1994). Common sub lethal effects are behavioral (e.g. swimming, feeding, attraction-avoidance, and prey-predator interactions), physiological (e.g. growth, reproduction, and development), biochemical (e.g. blood enzyme and ion levels), and histological changes (Sheehan *et al.*, 1984).

Increasing distribution of metals and metal compounds in the environment raises increasing concern for ecotoxicological effects. Reports of metal intoxication are common in plants, aquatic organisms, invertebrates, fishes, sea mammals, birds and domestic animals. Mercury poisoning from consumption of fish containing high levels of methyl mercury (Minamata Disease) and cadmium poisoning from consumption of rice grown in soils contaminated with cadmium from industrial discharges (*Itai-Itai* disease) are examples of human consequences from environmental pollution.

A toxicologically important characteristic of metals is that they may react in biological systems by losing one or more electrons to form cations (Vouch, 1986a). It is often the case that the nonessential toxic metals mimic essential metals and there by gain assess to and potentially disrupt key cellular functions. This can also account for bioaccumulation of toxic metals.

Chemically metals in their ionic form can be very reactive and can interact with biological systems in a large variety of ways. In this regard, a cell presents

numerous potential metal-binding ligand. For instance, metals like cadmium and mercury readily attach to sulfur in proteins as a preferred bio-ligand. Such adventitious binding is an important chemical mechanism by which exogenous metals exert toxic effects that can result re-arrangement that impairs the function of biomolecules (Kasprzak, 2002). An example is the inhibition of enzyme activity by metal interaction at sites other than the active center such as the inhibition of heme synthesis by lead. The inhibition of biologically critical enzymes is an important molecular mechanism of metal toxicology.

The metals can show more specific forms of chemical attack through mimicry. In this regard the toxic metals may act as mimics of essential metals, binding to physiological sites that normally are reserved for an essential element. Owing to their rich chemistry, essential metal control, or are involved in, a variety of key metabolic and signaling functions (Kasprzak, 2002, Cousins *et al.*,2006). For example for mimicry is the replacement of zinc, is a mechanism of toxicity for cadmium, copper and nickel. Thallium mimics potassium and manganese mimics iron as a critical factor. Molebdate, selenate and chromate mimic sulfate and can compete for sulfate carriers and in chemical sulfation reactions (Bridges and Zalpus, 2005). Organometallic compounds can also act as mimics of biological chemicals. As for example methyl mercury which is transported by aminoacids as organic anion transporters (Bridges and Zalpus, 2005).

Another key chemical reaction in metal toxicity is metal-mediated oxidative damage. Many metals can directly act as catalytic centers for redox reactions with molecular oxygen or other endogenous reactions with molecular oxygen or other endogenous oxidants, producing oxidative modification of biomolecules such as proteins or DNA. This may be key step in the carcinogenesity of certain metals (Kasprzak, 2002). Metals in their ionic form can be very reactive and form DNA and protein adducts in biological systems. Metal can also induce an array of aberrant gene expression, which in turn, produces adverse effects.

1.4 CADMIUM TOXICITY

Among the toxic heavy metals, major toxic metals are cadmium and lead. Cadmium is a toxic non essential transition metal discovered in 1817. Cadmium is a

xenobiotic placed on the black list of the majority of the International Conventions of pollution because of its cytotoxicity, its genotoxicity, its potential of bio-accumulation and its persistence (Taylor, 1983). This metal is typically found in ores with other metals, and is commercially produced as a byproduct of zinc and lead smelting, which are sources of environmental cadmium (Miramand *et al.*, 2000).

Cadmium has been recognized as one of the most hazardous environmental contaminant. It is considered more toxic than either lead or mercury. It is toxic at levels one tenth that of lead, mercury, aluminum or nickel (Wilson, 2008). Although its concentrations in the aqueous environment, both in water and in sediment are low, several fold enrichment is observed in the bivalve. According to USPHS 1997 and WHO 1992 standards permissible levels of cadmium in the fresh water and drinking water is less than one microns/liter and the same in the river sediment and soil is 1.0 mg/kg and 0.01 -2.0 mg/kg respectively.

Cadmium has a high solubility in sea water (up to 1000 ppm) compared to other metal pollutants, which further aggravates the toxicity of this metal to the aquatic fauna and flora. The major sources of this metal are mining and metallurgical operations, refining of zinc, lead and copper, electroplating industries, automotive tyres, pesticides, fertilizers, petrochemicals, fly ash, paints, plastics, photography, textile printing, batteries, leather tanning and sewage sludge. As many of the industries listed above exist in the state of Kerala, the chances of cadmium pollution in the nearby water bodies can not be ruled out. Moreover, the presence of cadmium in the wastes from a multinational company in Palakkad district of Kerala had raised hue and cry among the public in recent years. Thus the potency of cadmium as an environmental pollutant is an established fact and an understanding of the biological effects of this metal on fishes can be of help in identifying the pollution problem at an early stage.

1.5 LEAD TOXICITY

Lead is non-biodegradable toxicant, mainly occurs in the environment as an inorganic, metallic and organic lead forms. Metallic lead is resistant to corrosion and can combine other metals to form various alloys. Inorganic lead compounds are

used as pigments in paints, dyes and ceramic glazes. Organolead compounds were used as gasoline additives. Lead alloys are used in batteries, shields from radiation, water pipes and ammunition. Environmental lead comes mainly from human activity and is listed as top toxic substance (ATSDR, 2005c). The recommended standard value of environmental lead level is 0.05mg/l according to the world Health Organization (WHO). Recent studies suggest that there is in fact no level exposure that can be considered safe (Environment Impact Assessment Report, 2006)

Lead exposure in children still remains a major health concern. Lead – containing paint is a primary source of lead exposure in children that is the hand to mouth transfer of lead containing paint chips or dust from floors of older housing (Manton, *et al.,* 2000). Lead in household dust can also come outside of the house and may be related to lead in neighborhood soil (Von Lindren, *et al.*, 2003). The major route of exposure for the general population is from water and food. The leaded fish sinkers or pellets lost in the bottom of lakes and river banks can be mistaken for stone and ingested by birds causing adverse effects including health (De Francisco *et al.*, 2003). Main sources of lead pollution of aquatic ecosystems are the industrial discharge, atmospheric fall out and sewage effluents.

1.6 EFFECT OF HEAVY METAL POLLUTION

Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosyliene and Janakaite, 2006; Farombi, *et al.*, 2007). Among animal species, fishes are the inhabitants that can not escape from the detrimental effects of these pollutants (Olaifa *et al.*, 2004). Fish are widely used to evaluate the health of aquatic ecosystems because pollutants build up in the food chain and are responsible for adverse effects and death in the aquatic systems (Farkas, *et al.*, 2002). Fish is one of the most sensitive animals as bioindicators (Hybia, 1982; Storelli and Macrotrigiano, 2001). Research of Brumbaut *et al.*, (2005) point at the opportunity of using fish blood in biomonitoring.

Heavy metals alter the physiological activities and biochemical parameters both in tissues and in blood (Basa and Rani, 2003). Exposure to heavy metals

evokes several behavioral, physiological and biochemical changes that appear to be closely related. To counteract any stress, energy reserves, which might otherwise be utilized for growth, and reproduction will have to be diverted towards enhanced synthesis of detoxifying ligands (metal binding proteins, granules) or expended in order to maintain an elevated efflux of metal. Consequently, various enzymes related to energy metabolism alter their activity pattern depending on the nature of stress. Excess energy is required to carry out defensive behavioral responses that help animal to adapt and survive. This confers some confidence in quantifying metabolic changes in the energy parameters, and related enzyme activities as integrated markers of healthy physiological status. Toxic substances can injure gills, thus reducing the oxygen consumption and disrupting the osmoregulatory functions of aquatic organisms (Ghate and Mulherkar, 1979; Saravana *et al.*, 2000). Liver has the ability to degrade toxic compounds, but its regulating mechanisms can be overwhelmed by elevated concentrations of these compounds, and could subsequently result in structural damage (Brusle *et al.*, 1996). The heavy metal cadmium deposit in internal organs and impaired copper metabolism contributed to mortalities in the cadmium exposed group. The fish will ingest heavily polluted food until toxicity occurs. Wild fish may therefore be at risk from contamination after pollution incidents.

Chronic exposure of trout and carp to nickel, zinc, copper or chromium has been reported to suppress to a variable extent the primary humoral response to macrophage. Copper was found to cause immuno suppression of antibody producing cells in rainbow trout when tested *invitro* (Khangarot *et al.*, 1991) and in air breathing catfish *invivo* in a dose dependant manner along with depressed phagocytic activity of spleen and kidney macrophages, and suppression of T cell activity as indicated by prolongation of allograft rejection time (Anderson *et al.*, 1989). Defense against internal infections can be compromised by prolonged exposure to copper (Malins *et al.*, 1988).

Different concentrations of copper or zinc have been reported to cause dose – dependant suppression of kidney lymphocyte numbers and natural cytotoxic cells (Merchant and Packer, 1983). Copper caused a marked decrease in macrophage

activity both *invitro* and *invivo*, but zinc caused a modest increase in macrophage activity under the same conditions. In a study, exposure to copper reportedly caused a strong inhibition of the phagocytic response. However, Cadmium caused an initial stimulation followed by a variable decrease (Roubal, 1988).

Cadmium causes both, immunosuppression and immunostimulation in mammals depending on a variety of factors. T cell activities are usually suppressed whereas the effects on B cells are more varied. A concentration of 10 - 12 g/ml is about half the LC_{50} of cadmium. This concentration has been reported to cause inhibition of serum antibody titres in one species of fish but six fold stimulation in another (Newman and MacLean, 1974). A concentration of 0.7 or 3.6 g Cd/L has been reported to cause suppression of T lymphocyte function but enhancement of antibody response to bacterial challenge (Nielsen *et al.*, 2001). Cadmium can have a marked effect on differential leukocyte counts in fish. A dose-dependant three-fold increase in neutrophils and decrease in lymphocytes has been reported (Morra, 1993). Lesions in hematopoietic areas of lymphoid organs following exposure to cadmium have been reported (Plumb and Areechon, 1990). Elevated cortisol may be a primary mechanism for immune system suppression in fish exposed to a variety of pollutants. Most fish exhibit an elevated plasma cortisol level in response to nearly any stressor. However, cadmium alone among the metals fails to induce this hormonal change.

1.7 BIOMARKERS OF ANIMAL HEALTH

Physiological biomarkers may identify effects at a tissue/organ before they are apparent at a clinical/pathological level. Biological markers, or biomarkers, are observable properties of an organism that indicate biochemical components, structure or function and that can be measured biologically. Biomarkers are measures of sub organismal responses in organisms or exposed biological systems which can demonstrate exposure to, or the effects of, environmental contaminants (Peakall, 1992). Over the past two decades, the science of biomarkers has advanced considerably (SETAC, 1992), and there is a worldwide trend to supplement chemical and physical parameters with biomarkers in marine pollution monitoring (ANZECC, 1992). A variety of molecular, biochemical, physiological, histo-

cytopathological, organismal, population and community responses may be used to identify exposure to certain chemicals, provide information on spatial and temporal changes in the concentration of contaminants, and indicate environmental quality or occurrence of adverse ecological consequences (Au, 2004). In general, responses at lower biological organization level (eg. Molecular, physiological and biochemical responses) are more specific, sensitive, reproducible and easier to determine. Histo-cytological responses are relatively easy to determine, and can be related to health and fitness of individuals which, in turn, allows further extrapolation to population / community effects. Among the various biomarkers haematological, biochemical and histopathogical biomarkers are discussed in this work.

Qualitative and quantitative variations in haematological parameters including the red blood cell (RBC) and white blood cell (WBC) numbers, cell proportions of leukocyte, the amount of haemoglobin (Hb) and the size of the RBC and WBC are the most significant findings as regards diagnosis. On exposure to environmental pollutants especially heavy metals cause changes in the blood characteristics in fishes by stress. These indices have been employed in effectively monitoring the responses of the fish to the stressors and thus its health status under such adverse conditions. The role of blood parameters as biomarker in the assessment of the health status of fish is understood by the observation of Omoregie (1998) who noted the possibility that changes in the blood will reveal conditions within the body of the fish long before any outward manifestation of diseases.

Histopathological changes have been widely used as biomarkers in the evaluation of the health of fish exposed to contaminants, both in the laboratory (Wester and Canton, 1991; Thophon *et al.*, 2003) and field studies (Hinton *et al.*, 1992; Schwaiger *et al.*, 1997 and Teh *et al.*, 1997). One of the great advantages of using histopathological biomarkers in environmental monitoring is that this category of biomarkers allows examining specific target organs, including gills, kidney and liver, that are responsible for vital functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish (Gernhofer *et al.*, 2001). Furthermore, the alterations found in these organs are normally easier to identify than functional ones (Fanta *et al.*, 2003), and serve as warning signs of damage to animal health (Hinton and Lauren, 1990).

A clear understanding of heavy metal toxicity on the aquatic biota demands detailed investigations on the sub lethal effects of heavy metal at various concentrations which are more realistic with reference to environmental concentrations. Studies of sub lethal effects of metal in fish aim at analyzing the biological responses of an organism to metal exposure when the responses are quantified it forms a basis for bioassay procedures. Hence an attempt was made to study the sub lethal effects of cadmium and lead on the haematological, biochemical and histopathological alterations on the fish *Etroplus maculatus* in addition to the normal variations on the haematological parameters.

1.8 TEST ANIMAL

Etroplus maculatus (Bloch, 1795)

Scientific classification

Kingdom		Animalia
Phylum		Chordata
Class		Actinopterigii
Order		Perciformes
Family		Cichlidae
Sub Family		Etroplinae
Genus		Etroplus (Georges, Cuvier, 1830)
Species		*Etroplus maculatus*
Synonyms	-	Chaetodon maculatus
Commom name	-	Red chromide, Orange chromide, spotted etroplus

Etroplus maculatus (Pallathi in Malayalam) is an Indian Cichlid is selected to carry out the experimental study. *Etroplus maculatus* is an economically important food fish. This are traditionally considered as suitable cultivable species. It is caught from the wild and reared as an aquarium fish. They occur along the coastal tracts of peninsular India and are very common in the rivers, ponds, paddy fields, canals, lakes and estuaries of Kerala, southern India and Sri Lanka.

Etroplus maculatus is a small fish grow up to length of maximum of 8 cm and weigh up to 8 gms. It has large spot on the body. They show sexual dimorphism. Female fishes are smaller than the males and are less colored. Males are colored bright having red color on the fins. In the breeding season these fishes are shiny yellow otherwise grayish with reddish region in the belly.

The fish is generally peaceful and can be kept in community tanks if enough hiding places are present. The fishes are highly sensitive to sudden changes in the water chemistry especially juveniles often die if we carry out water changes in the tank during experiment. They live comfortably in the water having temperature ranges from 24 – 26 oC and in the pH range 6- 7.5. Higher temperature up to 30 o C induce couple to breed. The females lay 200-300 eggs on the surface of the water plant. Both parents take care of fry. The fry react to the black pelvic signal from the parent.

The stomach content includes insect larvae, zooplankton and small crustaceans. In the laboratory they feed well on bits of earth worm and boiled rice. Mouth opens wide when it is protruded and closes when it is retracted. Feeding is affected by protruding and retracting the mouth. This observation shows that the fish is a surface feeder or a column feeder and predominantly carnivorous. This species co-occurs throughout its range with in the green chromide (*Etroplus suratensis*). Orange chromide prey on the eggs and larvae of *Etroplus suratensis* and act as a cleaner fish removing parasite from the larger *Etroplus suratensis.*

Fishes are one of the most important members of the aquatic food chain, and through them some toxicants may reach human beings as well. The selection of organisms for toxicity test is mainly based on certain criteria like its ecological status, position within the food chain, suitability for laboratory studies, genetically stable, uniform populations and adequate background data on the organism (Buikema *et al.*, 1982). The species selected for the present study *Etroplus maculatus* satisfy most of the above protocols. Rechten (1980) opined it as a laboratory favorite of fish researchers. However, there are difficulties in the rise of fishes for pollution assessment impact. Most important of these is our limited understanding of the mechanism of toxicity. The interpretation of the significance

or specificity of a measured biological response could there for become difficult. Not withstanding these limitations, attempts have been made to the normal haematology and to analyze the impact of heavy metal at realistic levels to the experimental media, on the haematology, and enzymatic activity and histology of *Etroplus maculatus*.

1.9 COLLECTION SITE

Specimens of *Etroplus maculatus* employed in the study, were collected from the waters of Kadamakkudy. This region located on the lower reaches of Periyar River and the northern part of Cochin back water system. The Cochin estuary/ Cochin back waters, one of the largest tropical estuaries of India (Area - 256km2, 9degree 40'-10degree 10'N; 76degree10'-76degree 30'E), is facing gross pollution problems following the release of untreated effluents from industries and domestic sectors (Balachandran, *et al.*, 2006). The major polluting industries in the region include a fertilizer plant, an oil refinery, rare earth processing plant, mineral and rutile plants, zinc smelter plant, an insecticide factory and an organic chemical plant (Balachandran, *et al.*, 2006).

River Periyar has been performing a pivotal role in shaping the economic prospects of Kerala. The River Periyar, the longest river of the state (PWD, 1974; CESS, 1984) is considered to be the life line of Central Kerala. It originates from the Sivagiri peaks of Sundaramala in TamilNadu. The total length is about 300 Kms. During its journey to Arabian Sea at Cochin the river is enriched with water of minor tributaries like Muthayar, Perunthuraiar, Chinnar, Cheruthony, Kattappanayar and Edamalayar at different junctions. At Alwaye, the river bifurcates into the Mangalapuzha branch and the Marthandavarma branch. The former joins river Chalakudy and finally drains into the Lakshadweep Sea and the latter bisects the industrial belt at Eloor (Industrial Hot spot) before discharging into the backwaters adjoining the Arabian Sea (KSPCB, 1981). The major industries and settlements are in the lower reaches, especially in the Alwaye, Ernakulam belt. There are number of islands in the lower reaches of the basin. Kadamakudy is one of the islands.

Angamaly to Kochi is the most industrialized zone of the Periyar river basin. There are 50 large and medium industries and over 2500 small scale industries in this region. The industries located in Edayar-Eloor area consume about 189343 cum water per day and discharge about 75 % as used water along with large quantity of effluents and pollutants. The major types of these industries are fertilizers, pesticides, chemicals and allied industries, petroleum refining and heavy metal processing, radioactive mineral processing, rubber processing units, animal bone processing units, battery manufactures, mercury products, acid manufactures, pigment and latex producers etc. The wide spectra of pollutants that adversely affect the natural environmental quality of the water of the river include toxic and hazardous materials such as heavy metals, phenols, hydrocarbons, pesticides, radionuclide, and ammonia, and phosphates, domestic and untreated waste water. Etc. All this are discharged into the Periyar River along with the effluent from the factories and all this are carried to the Cochin back water systems. (KSPCB, 2001). Kadamakudy region is, however, located far from this industrial belt, and is considered as pristine area of Cochin backwaters.

The concentration of heavy metals lead and cadmium in the water samples of the collection site was recorded as 0.121mg/l and 0.026 mg/l and from the sediment sample lead is 10.25mg/l and cadmium is 7.17 mg/l.

Though several early workers dealt with taxonomy and physiology including food and feeding, reproductive behaviour, ecology, biology and culture aspects of these fishes. Much more remain to be known about the haematology, heavy metal toxicity and histopathology of these fishes. The present work attempts to find out the normal variations in haematological parameters under normal environmental conditions and to assess the toxicity of heavy metals cadmium and lead on the stress responses in the test animal *Etroplus maculatus*

The thesis is organized into 7 chapters.

Chapter 1. General introduction and objectives of the study.

Chapter 2. Deals with Review of Literature.

Chapter 3. Describes the normal haematological parameters of *Etroplus maculatus*.

Chapter 4. Deals with the Leucocytes and related cells of *Etroplus maculatus*.

Chapter 5. Includes the Sub lethal toxicity studies on *Etroplus maculatus*. It specifies the effect of heavy metals cadmium and lead on the haematological parameters.

Chapter 6. Deals with the effect of sub lethal concentration of cadmium and lead on biochemical and enzymatic parameters.

Chapter 7. Deals with the histopathological studies, the effect of cadmium and lead on the tissues of liver, gills and kidney.

Chapter 8. Summary and conclusion. Salient features of the investigations are summarized in this chapter.

References are given at the end of the thesis.

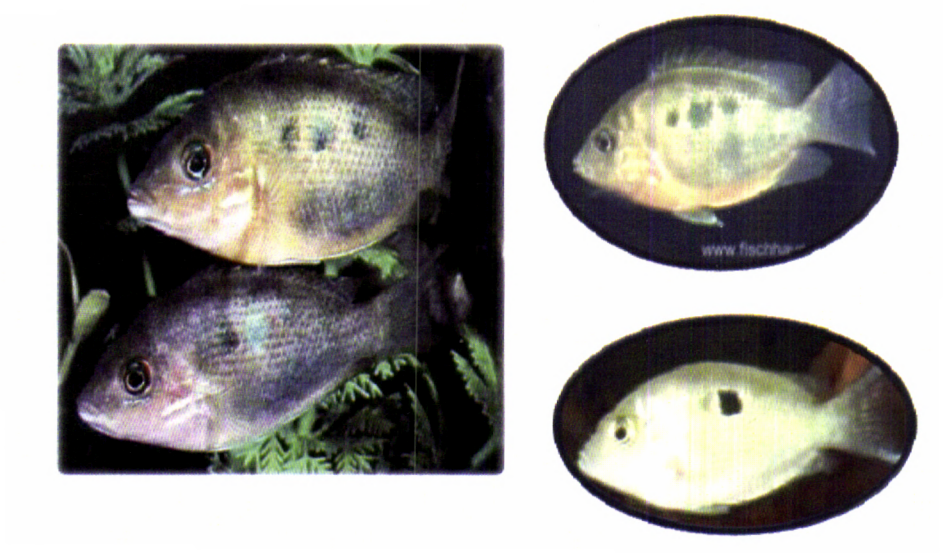


Fig: 1.1 Etroplus maculatus (Orange chromide, Pallathi)

Chapter 2
Review of Literature

REVIEW OF LITERATURE

2.1 NORMAL HAEMATOLOGICAL PARAMETERS

Scientific understanding of the haematology began with the contributions of Leeuwenhock and others in the seventeenth century with the miroscopic examination of blood (Wintrobe, 1985). Hematology is a diverse medical specialty, which perhaps more than any other discipline has made tremendous contributions to molecular medicine (Kaushansky, 2000).

On the perusal of literature, one can see that with in the last three decades, blood physiology has been one of the most sought after branches of life sciences all over the world. There are over 25,000 species of teleost living in fresh, brackish and salt waters. But the literature on blood covers only limited number of them.

Reference to the haematology and blood chemistry of tissues are included in the bibliography of Hawkins and Mawdesley-Thomas (1972). Blaxhall (1972) made a review of selected literature regarding the use of haematological techniques in fresh water fish pathology. Hille (1982) reviewed the literature on the blood chemistry of rainbow trout, *Salmo gairdneri* (Rich) based on experimental methods.

Despite fish haematology continues to offer a potential valuable tool, the progress to establish blood parameters is slow and literature on this subject is isolated and often incomplete (Kori-Siakpere *et al.*, 2005).

The haematological characteristics of a number of cultivable fish species have been studied with the aim of establishing normal value ranges, and any deviation from it may indicate a disturbance in the physiological processes [(Ranza-Paiva *et al* (2000), O'Neal *et al* (2001)]. Several of these studies were attempts to determine if significant variations from normal values of these parameters exist that could be attributable to some internal or external factors (Gabriel *et al.*, 2001)

Many investigations have done in fish blood to establish 'normal' values (Julieta, (1994); Rodrigues *et al.*, (2003); Ibrahim Orun *et al.*, (2003); Rehulka and Adamec (2004); Cazenave *et al.*, (2005); Kori-Siakpere *et al.*, (2005); Tavares-Dias and Moraes, (2006); Adebayo *et al.*, (2007); Rey Vazquez and Guerrero (2007); Mevlut Aras *et al.*, (2008)).

Hickey (1976) pointed out that various factors affect the haematological values in fishes, as in higher vertebrates (Schalm, 1967). According to Svobodova *et al.* (1994) blood parameters values are altered by the variables such as season, age, sex, dietary state, disease, pollutants, environmental parameters and stress.

Preston (1960) and Mahagen *et al* (1979) observed seasonal fluctuations in haematological parameters while Smith (1977) noted a correlation of MCV to weight which ranged from highly negative to highly positive based on the season. Seasonal variations affect various blood parameters in many of the teleost. Ibrahim *et al.*, (2003) indicated that blood parameter levels of three cyprinid fish species in warm months were significantly different than those measured in cold seasons. It was reported that the lowest Hb was found with in summer in tench (Guijarro *et al.*, 2003) and in mirror carp (Sahan and Azizoglu, 2000). Studies on *Tenualosa ilisha* by Jawad *et al.*, (2004) showed that the seasonal variation in haematological parameters is similar in both sexes. Mevlut *et al.* (2008) reported that there is variation in the haematological parameters of *Leuciscus cephalus* in the prespawning and postspawning periods. The highest value in RBC, Hct, MCV and WBC were found in May and summer (Pre spawning period) and the lowest in cold seasons (autumn and winter). An analysis of the results of the erythrocyte and leukocyte series, thrombocytes and phagocytic activity in *Centropomus parallelus* related to seasonal cycle showed statistically significant differences (Antenor *et al.*, 2009).

Dacie and Lewis (1991) reported that gender has a great influence on haematology of fish: however, sex related variations in various haematological values of fishes are scant (Haws and Goodnight, 1962; Banerjee, 1966; Poston, 1966; Snieszko *et al.*, 1966; Mulcahy, 1970). While Kori-Siakpere and Egor (1997) observed differences in haematology for different sexes of *Clarias buthupogon*.

Studies on sexually matured gold fish (*Carassius auratus*) (Summerfelt, 1967), brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo gairdneri*) (Sniezsko, 1960) showed that males consistently had higher packed cell volume values than the females and this has been proposed as means of sexing fish. While Etim *et al* (1999) did not observe any difference between male and female of *Chrysicthys nigrodigitatus*. Clement (2002) pointed out that the haematological response in male was lower than that of females in Rainbow trout from the wild , exposed to trapping and handling stress. From the studies of Ibrahim *et al.*, (2003) it is clear that the number of total leucocyte, neutrophil and monocyte levels were higher in female fish, especially in reproduction season, than in male fish. Levels of erythrocyte, hemoglobin and hematocrit were high in male fish in an annual period. Result showed there was no gender effects on erythrocyte indexes (MCV, MCH, and MCHC) eosinophil and thrombocyte levels. According to Jawad *et al.*, (2004) males showed a higher haematocrit value than females, independently to body length in *Tenualosa ilisha*, Indian shad. Akinrotimi *et al.*, (2007) observed in haematology of *Sarotherodon melanotheron* and Ezeri *et.al* (2004) in *Clarias* , with the female had higher value of Hb, Hct, WBC, MCHC, MCH, MCV, thrombocytes, neutrocytes and monocytes than the males before and after acclimation. Antenor *et al.*, (2009) observed that haematological results of fat snook (*Centropomus parallelus*) did not show significant difference between males and females.

The general trend in the relationship between blood hematocrit and body length is the longer the fish, the higher the hematocrit in *Cyprinus carpio* (Murachi, 1959) *and Clarias batrachus* (Joshi and Tandon, 1977). Jawad *et al.* (2004) postulated that haematocrit had a polynomial relationship to the length of fish, *Tenualosa ilisha*. Fish in the smallest size group had the lowest values of haematocrit. This increased as the fish length increased up to a certain level, after which no further increase was noted.

There may be a certain relationship between haematological values and behaviour in fish (Hall and Gray, 1929; Gray, 1946) since migratory and lotic environmental species have the highest values. According to Putman and Freel (1978) packed cell volume is a major haematological characteristic that change with

fish activity. Weels *et.al* (1980) stated that migratory fish have high hemoglobin rate which may be explained by the need for high energy waste. Val *et al* (1985) stated that there is no significant difference in Hb values of *Hypostomus regani* of lotic and lentic environments.

Blood attributes of fishes such as the size and number of erythrocytes and concentration of haemoglobin vary with ambient concentrations (Ytrestoyl, *et al.*, 2001). Blood parameters such as haematocrit, haemoglobin concentration and RBC count re related to environmental factors such as water temperature and salinity (Graham, 1997). The haematological parameters were positively affected by water temperature and gonadosomatic fluctuations (Martinez *et al.*, 1994; Frangioni *et al.*, 1997; Collazos *et al* 1998; Guijarro *et al.*, 2003). According to Fernandes and Mazon (2003), haematological parameters are closely related to the response of the animal to the environment, an indication that the environment where fish lives could exert some influence on the haematological characteristics (Kori-Siakpere, 1985). Bayir (2005) suggested that WBC was affected by some factors such as water temperature and reproduction period. Contrary to other haematological parameters; Hb, MCH and MCHC were quite negatively correlated with temperature changes and these parameters have no correlation with GSI. Denson *et al.*, (2003) and Anyanwu *et al.*, (2007) observed a reduction in the value of Hb and RBC in *Rachycentron canadum* and *Sarotherodon melanotheron* exposed to various degree of salinity.

Changes in the blood characteristics of *Clarias gariepinus* caused by stress because of exposure to environmental pollutants, diseases or attack by pathogens have been studied by number of authors (Onusiriuka and Ufodike, 2000; Ezeri, 2001; Gabriel *et al.*, 2001). Saravanan *et al.*, (2003) observed an increase in RBC and WBC contents in fish from polluted environments. Silveira-Coffigny *et al.*, (2004) and Simonato *et al.*, (2007) reported a decrease in RBC numbers and hematocrit in fish exposed to pollutants. Moiseenko (1998) reported an increased response of white blood cells (WBC) in fish, *Coregonus lavaretus*. Hematocrit percentage was greater in fish from the polluted site (Tavares-Dias *et al.*, (2002); Ranzani-Paiva *et al.*, (2005) and Gabriela *et al.*, 2009). The sex specific response in

haematological status of fish, when exposed to any external stressor according to Barton (1997) may be linked to altered sex steroid hormones which act as modulators of the stress response in fishes.

The result from the haematological studies on winter Flounder by Ohn and Kneeland (1992) indicated that most diseased fish had significantly lower levels of hemoglobin, erythrocytes and hematocrit than healthy fish; blood deficiencies were more frequent among juveniles than adults. This result indicated that the disease had severe consequences for this species.

It is reported in some studies that experimental haemoflagellate infections of salmonids and cyprinids caused some changes in the haematological parameters and that anaemia was a major clinical sign (Steinhagen *et al.*, (1990); Zuo and Woo., (2000)). Horton and Okamura (2003) suggest that a decrease in erythrocyte and hematocrit values and an increase in leucocyte count may occur in infested fishes. Rehulka, (2002) and Martins *et al.*, (2004b) reported a decrease in RBC numbers and hematocrit in infected fish, while an increase in the numbers of leucocytes and neutrophils have been reported in parasitized fish (Sopinska, 1985; Silva-Souza *et al.*, 2000; Ghiraldelli *et al.*, 2006b). Akmirza and Remziye (2007) determined an increase for eosinophils in autumn while a decrease for neutrophils in spring in infected roach. It was also found that there were the differences in the erythrocyte count and percentage of haematocrit seasonally.

External stressors like acclimation have great influence on the haematology of fishes. Seth and Saxena (2003), Yaji and Auta (2007) observed a decrease in haemoglobin, Haematocrit and RBC counts on acclimation in *Channa punctas* and *Clarias gariepinus*. Although there were no significant differences between the blood characteristics of apparently healthy fish before and after acclimation, the values of some of the parameters (Hb, PCV, RBC, MCV, WBC and neutrophils) were higher in the acclimated *Clarias gariepinus* (Gabriel *et al.*, 2004) indicates that *Clarias gariepinus* after acclimation, males consistently had higher values of WBC, neutrophils and monocytes than the females, but the reverse was the case with lymphocytes. It appears then that males are more responsive to the stress of acclimation than the females. Akinrotini, *et al.*, (2007) observed the same decrease

in the values in *Sarotherodon melanotheron* on acclimation. Hattingh and Van Pletzen (1974) reported that decrease in the fish blood haemoglobin concentration and PCV. Stress due to capture had been reported by Hattingh (1976) to cause hyperglycaemia in fish.

In general, the number of erythrocytes correlates negatively with their size (Kuramoto, 1981; Beitinger *et al.*, 1985). Although some authors suggest no correlation between body size and haematological parameters such as Hct, erythrocyte size and number and hemoglobin concentration (Calder, 1984; Garland and Carter, 1994). Kori-Siakpere (1985) and Gabriel *et al.*, (2004) observed that there was wide variations in the Hb, PCV and RBC indices of *Clarias ishierensis* from the wild.

Bridges *et al.*, (1976) had demonstrated significant seasonal differences in red blood variables, occurring with relation to the reproductive activity of *Pseudopleuronectes americanus*. It is known that blood parameters can change depending on the maturation of the gonads (Joshi, 1982; Ranzani Paiva and Godinho, 1985). According to Joshi (1982), the lowest values of red blood parameters were usually recorded in spent fish, or during winters when the temperature was quite low and the food scare. Luskova *et al.*, (1995) observed variations of some parameters in *Chondrostoma nasus*, coincident with both spawning and water temperature.

Rehulka and Adamec (2004) reported that water temperature does not seem to be responsible for variations in haematology of rainbow trout and that diet, metabolic adaptations and activity were the probable causes of seasonal fluctuations in haematological parameters such as Hb, Hct, MCV, MCH and MCHC.

The result from the haematological studies on winter Flounder by Ohn and Kneeland (1992) indicated that most diseased fish had significantly lower levels of hemoglobin, erythrocytes and hematocrit than healthy fish; blood deficiencies were more frequent among juveniles than adults. This result indicated that the disease had severe consequences for this species.

Younger stages of RBCs in blood was reported by various authors. Boomker (1980) identified 3 stages of polychromatophilic erythrocytes and erythroblast in the

blood of *Clariuas gariepinus*. Joshi (1987) identified three types of erythroblasts in the blood of various fresh water teleosts. As the nomenclature of developing series of erythrocytes is still not clearly explained, it is not known whether Boomker and Joshi are describing the same types of cells. Joshi (1987) also reported the presence of microcytes, macrocytes, crenated red cells and enucleated erythrocytes in the blood of fresh water teleosts.

A size and weight related correlation to haematological factors has been observed in teleosts. Erythrocyte number and haemoglobin concentration in female *H.fossilis* increased with the body weight (Pandy *et al.*, 1976). Preston (1960), Haws and Goosnight (1961), Dube and Datta Munshi (1973) got similar results in Plaice, Channel cat fish and anabas respectively. In *Rita rita*, Pandey and Pandey (1977) observed an increase in RBC number, Hb concentration and ESR with an increase in weight. But PCV was found to decrease in this fish with an increase in weight. Smith (1977) suggested that small fish have low blood oxygen solubility in spite of high weight s specific oxygen consumption. Das (1965) discovered that the number of red blood cells and haemoglobin concentration tend to increase with length and age. Jawad *et al*, (2004) studied the relationship between haematocrit and some biological parameters of the Indian shad, *Tenualosa ilisha*.

Changes in haematological parameters due to unfavorable exogenous factors like poor water quality, overstocking etc. are indices of the ill health of cultivated fish. Regular monitoring of the haematological parameters of farmed fish can be used to enhance fish production. (Adebayo *et al*, 2007).

(Hrubec *et al*, 2000) has published some haematological reference intervals for hybrid tilapias (*Oreochromis niliticus* X *O. Mossambicus* X *O. aureus*). Gina Conroy and David.Conroy (2007) set standard haematological parameters for farmed Tilapia.

2.2 LEUCOCYTES AND RELATED CELLS

The study of fish blood and fish blood cells is not a new scientific discipline; this discipline is undoubtedly nearly a century and a half old. Yet it is surprising to note only "little historical background in recent texts on fish haematology

"(Srivastava, 1989). Study of vertebrate blood cells including those of fishes , particularly teleosts, attracted scientific attention as early as 1845 when Gulliver made a detailed study of the red blood cells in scorpion fishes. The first classic work on the nomenclature of fish leucocytes was that of Weinberg (1911). Later many works were carried out by different researchers to study the leucocytes of fishes. During the following ten years or so, though there was an apparent slacking of activity in this direction, a heightened interest was evident in the study of the plasma / serum chemistry of fish blood (Ellis, 1977). Detailed study of teleost blood cells, their identification, classification and nomenclature have also attracted during the first four decades of this century.

Several works on the characteristics of fish leucocytes were performed (Watson *e .al.*, 1963; Ellis, 1976; Finstad *e .al.*, 1964; Catton, 1951; Bielek, 1981). However; there are differences of nomenclature and classification among workers. In rain bow trout leukocytes, Yuki (1957) revealed lymphocytes, granulocytes and monocytes. Weinberg (1958) reported lymphocytes, thrombocytes, neutrophils and occasionally eosiophils and basophils. Suzuki (1984) revealed lymphocytes, thrombocytes, neutrophils and monocytes. In eel leucocytes, Sano (1957) reported lymphocytes, neutrophils and monocytes. But, Hoshina (1962) demonstrated the presence of lymphocytes, thrombocytes, neutrophils and monocytes Furthermore Mushiake *et al* (1985) reported that in copper exposure effect on phagocytosis, phagocytosis could not be distinguished from neutrophils, eosinophils and basophils.

Leukocytes are chiefly involved in specific and non-specific defence mechanisms. A number of enzymes, including peroxidases, phosphatases, phosphorylases, esterases and dehydrogenases have been found to be localized in leucocytes where they control and regulate antigen trafficking and thus assist in the defence mechanism. These enzymes have also been localized in piscine leucocytes. Garavini *et al.*,(1981) reported the presence of alkaline phosphatase and peroxidase in neutrophils of *Ictalurus punctatus*. (Douggett *et al.*. 1987) demonstrated the presence of nonspecific esterases and peroxidase in granulocyte type I of *Oreochromis mossambicus*, but only the nonspecific esterase was present in

monocytes and lymphocytes. Prasad and Radhakrishnan (1992) studied enzymes in the leucocytes of *Anguilla bicolor bicolor*. In 1995, Hammers studied the alkaline phosphatase and ASD chloroacetate esterase enzymes in monocytes of the peripheral blood of *Cyprinus carpio*. In Elophomorpha, Stomiformes and Acanthopterygians, Hine and Wain (1988a, b) identified the presence of alkaline phosphatase, alphanaphtyl acetate esterase and ASD in fine granulocytes of chimeras, and peroxidase in the eosinophils and neutrophils. A study of neutrophil and macrophage responses in rainbow trout (Afonso *et al.*, 1998) revealed that the macrophages of the respective species are peroxidase negative but esterase positive. Tavares-Dias (2006a) reported the presence of nonspecific esterase in the monocytes and peroxidase in the neutrophils of *Astronotus ocellatus*, *A. Nobilis*., *Hoplias malabaricus* and *Astynax bimacculatus*. Prasad and Sonia (2009) studied the haematology and leucocyte enzyme cytochemistry of a threated yellow catfish *Horabagrus brachysoma*.

As for the function of leucocytes, phagocytosis of the foreign material is one of the most important mechanisms which protect the body to the infection (Hatai, 1972). There are some works about phagocytosis of fish leukocytes (Ellis, 1976; Suzuki, 1984; Mushiake *et al.*, 1985, Ferguson, 1985; Mc Kinney *et al.*, 1979). Watson *et al* (1963) reported that experimentally induced bacterial infection in gold fish, neutrophils, eosinophils and macrophages containing bacteria were observed on the site of infection. Ellis (1976) observed that in plaice, monocytes and macrophages showed engulfment of colloidal carbon particles. However in Plaice, Ferguson (1976) reported that thrombocytes, rarely neutrophils and monocytes showed carbon uptake. Ms Kinney *et al* (1979) revealed that in gar, thrombocytes and macrophages exhibited phagocytosis. Suzuki (1984) reported that in rainbow trout and rockfish, thrombocytes, neutrophils and monocytes showed ingestion of bacteria. Thus there are differences of phagocytes not only among the different species but also in the same species. These differences may be caused by the method used for classification.

Saunders (1968) made differential blood cell counts of fifty species of fishes from the red sea. He listed the teleost leukocytes under the following categories,

hemoblasts, macrophages, lymphocytes, thrombocytes and granulocytes include eosinophils, heterophils and neutrophils.

Cannon *et.al* (1980) made an ultrastructural study of the leukocytes of the channel catfish *Ictalurus punctatus*. Barber et.al., (1981) made a light and electron microscopic observations on the blood cells of the Antartic ice fish *Chaenocephalus aceratus* Lonnberg. He could find that erythrocytes were rare and observed two types of granulocytes, lymphocytes, thrombocytes and monocytes or macrophages. Kunio Suzuki (1984) conducted a light and electron microscope study on the phagocytosis of leucocytes in Rockfish and Rainbow Trout. Richii Kusuda and Yuki Ikeda (1987) studied the classification of eel leukocyes. Studies on composition and ultrastructure of elasmobranch granulocytes have made by Hine and Wain (1987). Doggett and Harris (1989) studied the ultrastructure of the peripheral blood leucocytes of *Oreochromis mossambicus* and the phagocytic properties of the different leucocyte populations. Fujimaki and Isoda (1990) studied the fine structural study of leucocytes in the gold fish, *Carassius auratus*.

The cytochemical aspects of blood cells have been described in different species of fishes by Blaxhall and Daisley (1973); Barber and Westermann (1978); Caxton –Martins , (1979) ; Doggett ., (1978) and Zinkl *et al.*, (1991) have been described in different species of fishes by have studied this aspect for various species of fish.For thrombocyte. Imagawa *et al* (1989) and Ueda *et al.*, (1997) and ueda *et al* (2001) have described differences in their morphology. Hine *et al* (2006) and Prasad and Radhakrishnan (2006) studied the enzyme cytochemical characteristics of Indian eels, *Anguilla* species.

There is currently no data available on the morphological and enzyme cytochemical characteristics of leucocytes of *Etroplus maculates*. The aim of the present study reported here to identify the various types of leukocytes based on their morphological and staining properties and the localization of leucocytes enzymes which are not currently unavailable, thereby providing a basis for future comparative studies. In this study, morphological and staining characteristics of blood cells of brackish water fish, *Etroplus maculates* were revealed.

2.3 EFFECT OF HEAVY METALS CADMIUM AND LEAD ON THE HAEMATOLOGICAL PARAMETERS

Fish live in very intimate contact with their environment, and are therefore very susceptible to physical and chemical changes which may be reflect in their blood component (Wilson and Taylor, 1993). In fish, exposure to chemical pollutants can induce either increase or decrease in haematological levels.

The blood parameters have proved to be quite sensitive in laboratory experiments (Fromm, 1980; Giles *et al.*, 1984; Wood *et al.*, 1988a; Leino *et al.*, 1992; Holopainen and

Oikari, 1992; Van Dijk *et al.*, 1993). The study of the physiological and haematological characteristics of cultured fish species is an important tool in the development of aquaculture system, particularly in regard to the use in detection of healthy from diseased or stressed animal (Rainza-Paiva *et al.*, 2000; O'neal and Werich, 2001).

A survey of literature on heavy metal toxicity clearly shows that heavy metals cause several haematological and biochemical disorders both in laboratory animals as well as on aquatic organisms. The toxicity of lead, copper and other metals has been studied since mid 1920. (Quasim, 1923; Dilling *et al.*, 1923; Carpenter, 1927, Cardift, 1937; Ellis, 1937; Laurie *et al.*, 1938; Newton, 1944; Jones, 1947; Davidson, 1949; Fry, 1957; Cairns and Scheir, 1958; Imanishi *et al.*, 1959; Hynes, 1960; Lloyd., 1961; Zavon, 1964 ; Ray *et al.*, 1964; Sprangue (1970); Van Vuren, 1986; Lebedeva *et al.*, 1998; Vosy liene, 1999; Olifia *et al.*, 2002; Andharabi *et al.*, 2006; Shah, 2006; Adeyemo, 2008 and Mastan *et al.*, 2009).

Changes in the blood characteristics of *Clarias gariepinus* caused by stress because of exposure to environmental pollutants, diseases or attack by pathogens have been studied by a number of authors (Onusiriuka and Ufodike, 2000; Ezeri, 2001; Gabriel *et al.*, 2001.

Cadmium as a pollutant gained worldwide attention with the outbreak of 'itai-itai' disease in village on the banks of Jintsu River, Toyama Perfucture, and Japan in the year 1947. The name 'itai-itai' (meaning "oush-ouch") was so given in

accordance with the patients "shrieks" resulting from painful skeletal deformalities (Kobayashi, 1971). It is estimated that approximately hundred deaths occurred until the end of 1965. The cause of the was traced to the consumption of cadmium contaminated rice from fields irrigated with water from Jintsu river which was polluted with the effluents from a nearby Zinc mine. This gave an impetus to research on cadmium toxicity and at present, there is a plethora of information available on various aspects of cadmium toxicity.

The toxicity of cadmium is due in part to its competition with essential metal for binding sites and also its interference with sulfhydryl groups, which are essential for the normal functioning of enzymes and structural proteins. Cadmium blocks sulfhydryl groups in enzymes and competes for sites with zinc and calcium (WHO, 1971; Allen, 1994). An enzyme with a thiol group is a potential target for this pollutant (Romeo, 1991). Cadmium is one of the most harmful heavy metal to animals and has a particularly long biological half-life (Biegniewska. *et al.*, 1992) and the potential for exposure has increased with increasing industrial use of this metal (Park *et al.*, 1994). Toxicity of cadmium to fishes has stimulated considerable interest in recent years (Sastry and Shukla, 1994). Results of the studies on toxic intracellular processes show that metals are transported through the biological membranes and interfere with biochemical functions. The stable state of cadmium in the natural environment is Cd 2+. Cd is an oxyphilic and sulphophilic element. It exists totally as the divalent species up to PH 8, in the absence of any precipitating anions such as phosphate or sulphide and begins to form Cd(OH) + at PH 9 (Mohapatra and Saha, 2000). Cadmium exposure has been established to induce cancer and circulatory diseases in laboratory animals and the international agency for Research on cancer has identified cadmium as a human carcinogen (Satoh-Masahiko. e*t al.* 2002).

Most aquatic organisms have the capacity of concentrating metals by feeding and metabolic processes, which can lead to accumulation of high concentrations of metals in their tissues. Metal interact with legends in proteins particularly, enzymes and may inhibit their biochemical and physiological activities (Passow *et al.*, 1961). Cadmium sensitivity and bioaccumulation capacity are greater in marine

invertebrates than in marine teleosts. This differences, which is explained in part by weaker diffusion barrier between sea water and internal organs, is correlated with a high density of cadmium channels in the plasma membrane of muscular and nervous tissue s of invertebrates (Daemers *et al.*, 1998)

The toxicity of cadmium was antagonized by the low concentration of manganese. The toxicity also varies with the various inorganic salts forms in which it is exposed. Studies have shown that other metals, vitamins, chelating agents and proteins diets which alter the physiological, biochemical and behavioural aspects in fish also influence cadmium toxicity (Sastry and Shukla, 1994)

The variation in the toxic nature of cadmium to different species of organisms is dependent upon a number of physiological characteristics. Species difference in sensitivity to cadmium toxicity exists between *Channa punctatus* and *Clarias batrachus*

(Rana and Singh, 1996). An enzymological observation reveals that *Clarias punctatus* is better equipped with conjugating enzymes than *Clarias batrachus* which makes it a more resistant species. Lyons *et al* (1996), using immortalized cell lines have showed that mammalian cells are more sensitive to cadmium than fish cells. However, human epithelial explants are less sensitive to cadmium compared to rain-bow trout tissue explants.

Living organisms have evolved defensive mechanisms to overcome the Cd 2+ toxicities and in eukaryotes cell sequester cd2+ as biologically inactive forms with cysteine rich peptides such as Glutathione (GSH), phytochelatins and /or metallothioneins (Mehra and Winge, 1991; Perego and Howell, 1997; Nies, 1999; Bruins *et al.*, 2000; Hall, 2002; Cobbett and Goldsbrough, 2002). The mechanisms by which mammalian cells protect themselves against this toxic metal ion are very complex and not well understood. (Perego and Howell, 1997; Zalups and Ahmed, 2003).

Some substances are found to reduce or nullify the toxic effects of cadmium. The most widely used technique for the removal of toxic elements involves the process of neutralization and metal hydroxide precipitation (Hiemesh and Mahadevaswamy, 1994) A membrane therapeutic drug " Essentiale" (Natterman,

FRD) was found to be effective in combating the cadmium induced structural and biochemical changes in the intestine of *Oreochromis mossambicus* (Kothari *et al.*, 1999). Another substances, Zeolite is also found to reduce cadmium toxicithy in *Heteropneustes fossilis* and *Oreochromis mosssambicus* (James and Sampath, 1999, James, 2000). Quicklime (Cao) has the property to reduce cadmium toxicity (Kaviraj and Dutta, 2000). Shaffi *et al.* (2001) have studied the efficacy of selenium and zinc to combat the cadmium toxicity in *Labeo rohita*. They are of the opinion that, selenium prevents cadmium induced necrosis, blood pressure, injury to pancreatic beta cells and induction of hepatoglucogenic enzymes. Zinc also neutralizes the toxic effect of cadmium. The protective action of humic substances and calcium on cadmium toxicity has been studied in Zebra fish (*Danio rerio*) by Mainett, *et al*; 2001). Adel Shalaby (2007) investigated the effect of EDTA on reduction of toxicity of cadmium for enhance the change of blood parameter and enzymes and to assess its impact on some physiological parameters of Nile Tilapia (*Oreochromis niloticus*). In the light of that work he found out that EDTA appears to be promising tool to control cadmium pollution in aquaculture.

Generally, aquatic toxicological research is being applied at higher levels of biological organization like populations, communities and ecosystem to monitor environmental effects, conduct hazard assessment and make decisions of a regulatory nature (Mayer *et.al.*, 1992, Varanasi *et.al.*, 1992). But the fact is that ecologically important effects have already occurred at the higher levels. So with a thorough understanding of the effects of the toxicants on various physiological indices, the problems, can be detected before it affects the ecosystem as a whole. The physiological indices generally affected by the pollutants include the hematological parameters, enzymes, serum proteins, glucose and glycogen levels, cholesterol and hormones.

Cadmium causes marked changes in various haematological parameters in aquatic organisms. Significant reductions of haematocrit, haemoglobin and red blood cell count with significant increase in lymphocytes were found in the cadmium exposed flounders *Pleuronectus flesus* (Sjobeck and Larrson, 1978). In a field investigation on perch, *Perca fluviatilis* from a cadmium contaminated river,

Sjobeck *et al.*, (1984) have reported that the lymphocyte count is 45 – 100 % higher than those from reference habitats. A slight anemia is also noticed in this fishes. A dose of 24 ppm of cadmium nitrate for 90 hrs causes significant decrease in erythrocyte count, hematocrit and haemoglobin content and an increase in erythrocyte sedimentation rate (ESR) in *Cyprinous carpio* (Reeia and Viswarajan, 1987). However, the leucocyte count, thrombocyte count and blood clotting time did not significantly change due to the exposure. Effect of 24 hr LC 50 concentrations of Cdcl2 on erythrocyte and its related parameters in *Anabas testudineus* has been studied by Banerjee and Kumari (1988). No significant change is noticed in shape, length, and breadth and erythrocyte surface area. Nucleus becomes oval from almost round shape. Total erythrocyte count haemoglobin content and packed cell volume (PCV) decrease significantly where as ESR, Mean Corpuscular Volume (MCV) and Mean Corpuscle haemoglobin (MCH) increase significantly. Morsy and Protasowick 1990) have observed that, cadmium bioaccumulation significantly raises erythrocyte count, haemoglobin content, haematocrit value and blood glucose, but decreases leukocyte count in *Cyprinus carpio* L. when exposed to acute concentration of cadmium (0.5 mg cd/ dm3 water) for 24 hrs. Tort *et al* (1990) have exposed dog fish, *Scyliorhinis caniculs* to a cadmium concentration of 50 ppm for 1, 2, 3, and 4 days and no differences were found in majority of haematological parameters, except for the significant increase in red blood cell counts. In *Anabas testudineus* a sublethal concentration of cadmium for 30, 45 and 60 days evoked a significant increase in erythrocytes count, leukocyte count and haemoglobin (Hb) concentration (Saravanan and Natarajan, 19991). Allen (1993) has studied the effects of acute exposure to cadmium chloride on the haematological profile of *Oreochromis aureus* (Steindachner). Plasma osmolarity is the most sensitive blood parameter affected before other parameters change and cadmium does not depress erythrocyte counts. Cadmium chloride both at week and strong dose level, produce haematological abnormalities in *Tilapia mossambica* (Aziz *et.al.*, 1993). An increase in leucocyte count, erythrocyte count, Hb, PCV and MCV have been recorded at a strong dose of cadmium chloride (10 ug/ 15 l) for 2 days. On extending this exposure for 7 days Hb, leucocyte count, erythrocyte count and MCH increase, where as PCV and MCV decrease. Fish

exposed to weak dose for a period of 7 days (2.5 ug/ 15 l) have shown an increase in Hb, leucocyte count, erythrocyte count , PCV, MCV and MCH. Mukherjee and Sinha (1993) have observed a marked decrease in Hb, haematocrit (Hct) value and total erythrocyte count along with an increase in MCV and MCH after two weeks of cadmium exposure in *Labeo rohita*. In American eel, *Anguilla rostrata*, after 8 weeks of exposure to 150 ug cd/l, there was a significant reduction in the total erythrocyte count, leukocrit and large lymphocytes were significantly increased, while the proportion of small lymphocytes falls. (Gill and Epple, 1993). Changes in erythrocyte organization on exposure to cadmium have been studied in gold fish, *Carassius auratus* by Houston *et.al* (1993). In fish exposed to 11 % of LC 50, total cell numbers and the incidence of cell division decreases while karyorrhexis increases. At a sublethal concentration of cadmium the total leucocyte count increases in fish, *Channa punctatus* (Sastry and Sachdeva 1994) where as total erythrocyte count decreases (Bala *et al.*, 1994). The major Carp, *Catla Catla* shows a drastic decline in total erythrocyte count, Hb, and PCV on exposure to cadmium (Vincent *et al.*, 1996). Thus the haematological alterations due to cadmium toxicity can not be generalized and is highly variable between as well as with in species.

Exposure to cadmium causes the most pronounced changes in the leucocytes ratio in peripheral blood of common carp compared to other metals in the order Cd> Pb> Cu>Hg, and neutrophils are the most sensitive type of blood cells (Erpunin and Korebeinik-ova, 1997). A study performed on Mozambique tilapia (*Oreochromis mosambicus*) revealed not only neutrophilia but also thrombocytosis (Ruparellia *et al.*, 1990). At the same time, exposure of rainbow trout (*Salmo gairdneri*) to cadmium did not reveal any changes in the leucocyte formula and in ratio of phagocyte cels (Thuvander, 1989).

Atef M. Al-Attar (2005) studied the changes in haematological parameters of the fish, *Oreochromis niloticus* treated with sublethal concentration of cadmium. Kori-Siakpere *et al.* (2006) studied the sublethal effects of cadmium on some selected haematological parameters of *Heteroclarias* (A hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*). Kori-Siakpere *et.al.* (2006) studied the sublethal effects of cadmium on some selected haematological parameters of *Heteroclarias*

(A hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*). Sweety Remyla *et.al.*, (2008) studied the influence of zinc on cadmium induced haematological and biochemical responses in a fresh water teleost fish *Catla catla*. Deepak Kasherwani *et.al.* (2009) observed the cadmium toxicity to fresh water catfish, *Heteropneustes fossilis* (Bloch). Jana Kovarova *et.al.* (2009) observed the effect of cadmium chloride on metallothionein levels in carp. Zabotkina *et.al.*, (2009) studied the influence of cadmium ions on some morphofunctional and immune-physiological parameters of Perch under yearlings. Osman *et.al.*, (2009) studied the field application of humic acid against the effect of cadmium pollution on cultured Tilapia *Oreochromis niloticus*.

Changes in the blood characteristics of *Clarias gariepinus* caused by stress because of exposure to environmental pollutants. diseases or attack by pathogens have been studied by a number of authors (Onusiriuka and Ufodike, 2000; Ezeri, 2001; Gabriel *et.al.*, 2001. Syed Lal Shah (2006) studied the haematological parameters in tench *Tinca tinca* after short term exposure to lead

Apart from cadmium and lead, other toxicants have been known to adversely affect fish haematology. Gobacher and Skaya (1977) observed some chronic effects of organophosphate insecticide on fish haematology. McKim *et.al.*, (1970) observed in the haematological parameters of the blood of brook trout (*Salvelinus fontalis*) after a short term and long term exposure to copper. The measurement of specific and biochemical changes in *Salvelinus fontalis* exposed for short periods to sublethal environmental stressors, which have provided a sensitive method for predicting the effects of chronic exposure on survival, reproduction and growth. Nair *et.al* (1984) studied the effect of titanium effluents on the peripheral haematology of Anabas testudineus. Haematological alterations have there for allowed for a relatively rapid evaluation of the chronic toxicities of a compound. Kori-Siakpere (1991, 1995) reported the chronic sub lethal haematological effects of copper in fresh water teleost, *Clarias isheriensis* and some alterations in haematological parameters in *Clarias isheriensis* exposed to sub lethal concentrations of water borne lead. Annume and Ahume (1998) observed sublethal haematological changes in mudfish, *Clarias gariepinus* when exposed to copper

and lead. Navaraj and Kumarajuru (2003) studied the effect of electroplating effluent on haematological parameters of *Oreochromis mosambicus*. Elena Tomova *et.al.*, (2008) observed the effects of zinc on morphology of erythrocytes and spleen in *Carassius gibelio*. Kori-siakpere and Ubogu (2008) studied the sublethal haematological effects of Zinc on the freshwater fish, *Heteroclarias* sp. (Osteichthyes: Clariidae). Maheswaran *et.al*, (2008) conducted the haematological studies of fresh water fish, *Clarias batrachus* (L.) exposed to mercuric chloride.

Atef M. Al-Attar (2005) studied the changes in haematological parameters of the fish, *Oreochromis niloticus* treated with sublethal concentration of cadmium. Kori-Siakpere *et al.* (2006) studied the sublethal effects of cadmium on some selected haematological parameters of *Heteroclarias* (A hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*). Kori-Siakpere *et.al.* (2006) studied the sublethal effects of cadmium on some selected haematological parameters of *Heteroclarias* (A hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*). Sweety Remyla *et al.*, (2008) studied the influence of zinc on cadmium induced haematological and biochemical responses in a fresh water teleost fish *Catla catla*. Deepak Kasherwani *et al.* (2009) observed the cadmium toxicity to fresh water catfish, *Heteropneustes fossilis* (Bloch). Jana Kovarova *et al.* (2009) observed the effect of cadmium chloride on metallothionein levels in carp. Zabotkina *et al.*,(2009) studied the influence of cadmium ions on some morphofunctional and immune-physiological parameters of Perch under yearlings. Osman *et al.*, (2009) studied the field application of humic acid against the effect of cadmium pollution on cultured Tilapia *Oreochromis niloticus*.

2.4 EFFECT OF HEAVY METALS CADMIUM AND LEAD ON THE ENZYMATIC PARAMETERS

There are studies examining the effects of sublethal chronic concentrations of heavy metals on fish and these studies aim at morphologic and biochemical variations in the organs of different species of fish (Davalli *et al.*. 1990; Bieniarz *et al.*, 1996; Lionetto *et al.*, 1998; Hollis *et al.*, 1999; Wong and Wong, 2000; Zhou *et al.*, 2001; Cavas *et al.*, 2005; Tyagi and Srivastava. 2005; Loganathan *et al.*, 2006)

Experiments that are done in the field of ecological toxicology (Vilella, *et al.* 2000) are oriented towards studying the variations in the haematological fish indicators. De la *et al.*, (2002). Studied the response of brain acetylcholinesterase activity in two fish species caged *Cyprinus carpio* and field captured *Cnesterodon decemmaculatus*. Mathan Ramesh (2006) assessed the impact of heavy metal cadmium on certain enzymes in a fresh water teleost fish, Cyprinus carpio. De la Torre *et al.*, (2007) assessed the pollution impact on biomarkers of effect of a fresh water fish. He selected biochemical and morphological variables as biomarkers. And effects assessed on adult female of *Cnesterodon decemmaculatus*.

Glycogen depletion in liver and muscle after toxic stress in aquatic animals has been reported by various authors like Shobha *et al.*, (1989); Bhavan *et al.*, (1997) Aguiar *et.al.*, (2004); and Hori *et al.*, (2006).Nakano and Tomlimson., (1967) reported an increased secretion of catecholamine under stress conditions in rainbow trout, enhancing the utilization of glycogen for energy production. This effect on glycogen levels appears to be linked, at least to some extent, to the detoxification mechanisms, indispensable for metabolism or degradation and elimination of the pesticides from the body. Grant and Mehrle (1973) gave a possible explanation of glycogen depletion in Endrin exposed Salmo gairdneri that, stress-induced secretion of catecholamine and glucocorticoids can lead to increased glycogenolysis. Reduction of nutritional reserves during starvation in either blood or tissues has been observed in several crustacean species, and has been used as an indicator of nutrient metabolism (Pascual, *et al.* 2006). Glycogen depletion and hyperglycemia have been observed in cadmium exposed fish and explained as an effect of cadmium on the hormonal regulation of the glucose level (Larsson and Haux, 1982; Sastry and Subhadra, 1982).

Fishes have a very little amount of carbohydrates; the next alternative source of energy is protein to meet the increased energy demand. Umminger (1970) and Narasimhan and Sundararaji (1971) observed lower and total protein content in Pundulus heteroclitus and Notopterus notopterus respectively, exposed to various kind of stressors. During stress condition, fish need more energy to detoxify the toxicants and to overcome stress. As reported by Reddy and Bashamohideen (1995)

and Singh *et al.* (1996) the depletion of protein fraction in liver, muscle and gonad tissues may have been due to their degradation and possible utilization of degraded products for metabolic purposes.

Gilbert and O'Connor (1970), Chang and O'Connor (1983) reported that lipids are alternate sources of energy for organisms particularly in stress conditions and accelerated hydrolysis of lipids occur in order to cope with increased energy demand in stressed conditions. Supportive observations have been made in the fish *S.mossambicus* exposed to methyl parathion and in Barbus chonchonius exposed to aldicarb by Rao and Rao (1981) and Pant (1987) respectively.

ALT and AST are widespread in animal tissues and they are of considerable importance in metabolic economy. Knox and Greengard, (1965); Watts and Watts, (1974); and Martin *et al.*, (1983) reported that Aspartate aminotransferases (AAT) and Alanine aminotransferases (ALAT) are functioning as link between carbohydrate and protein metabolism catalyzing the interconversion of strategic compounds like aspartate and α-ketoglutaric acid to oxaloacetic acid and glutamic acid and α-ketoglutarate and alanine to glutamic acid and pyruvic acid respectively. Transaminases are intracellular enzymes which exist only in small amounts in serum. Transaminations might be of particular importance under conditions that impose a heavy drain on the animal's store of metabolites. As per Philip *et al.* (1995) elevation in the transaminases indicates the utilization of amino acids for the oxidation or for glucogenesis and is used to determine liver damage. Friedman *et al.*, (1996) and Henderson *et al.*, (1983) concluded that plasma activity concentrations of AAT and ALAT are the most commonly used biochemical markers of hepatocellular necrosis. La Due *et .al.* (1954) observed that these enzymes may leak into the plasma following reservoir tissue damage or dysfunction. Hence, the assay has become an indispensable tool in the clinical determination of the pathological conditions of the reservoir tissues and organs. Sastry and Sharma, (1980) and Das (1998) reported an elevation of brain acid phosphatase activity in stress induced Channa punctatus and *Labeo rohita* respectively.

According to Rajyasree and Neeraja, (1989), Oluah (1998, 1999), Balint *et al.* (1997) and Zikic *et al.* (2001) the increase in ALAT and AAT indicate the tissue damages in liver, kidney, and gill. Philip and Rajasree (1996) reported that alanine and aspartate transaminases function as biochemical stress biomarkers and their alteration allows identification of damage in different organs such as the liver. The involvement of lysosomal system in the metabolism of many metals, either through sequestration and binding of metals within the lysosome or as a target of their toxicity has been reviewed by Moore and Stebbing (1976). Rajalakshmi and Mohandas (2005) suggested ACP as a reliable marker tool for the biological assessment of metal pollution. ALP, which is sensitive to metals, gives a better picture of the general metabolic condition of the organisms (Regoli and Principato, 1995; Xiao *et al.*, 2002). Intestinal and serum ALP activities were stimulated at 10 μM Cu exposure (Alti and Canli, 2007) in the fresh water fish, *Oreochromis niloticus* (O.niloticus).

Pelgrom *et al.*, (1995) observed that, increase in the activities of functional enzymes in the blood serum and tissues of fish exposed to toxicants could be attributed to cell membraneous system damage leading to changes in membrane permeability and intercellular metabolism. Investigations have shown that changes in carbohydrate metabolism in fish induced by the severe stress resemble the changes displayed by higher vertebrates like mammals. As per Everse and Kaplan (1973) Lactate dehydrogenase forms the centre of a carefully balanced equilibrium between catabolism and anabolism of carbohydrates. Ramesh *et al* (1993) given that lactate dehydrogenase (LDH) is a marker of tissue damage and its increased level is reported in liver necrosis.

Dela *et.al*, (2000) conducted biomarkers assessmet in juvenile *Cyprinus carpio* exposed to waterborne cadmium and he measured parameters like gill ATPases, brain acetylcholinesterase (AchE), liver glumate oxaloacetate (GOT) and glutamate pyruvate (GPT) transaminases, protein content in liver, gills and brain.

2.5 HISTOPATHOLOGICAL EFFECT OF HEAVYMETALS IN FISH

Pathology, as a standard part of environmental monitoring programmes on effects of pollution, was approved by Moore (1980); Balouet and Poder (1981) and Couch (1985). Histopathology is the study of lesions or abnormalities on a cellular level in fishes. Histopathological examination is widely recognized as a reliable method for disease diagnosis and for assessing acute and chronic effects of exposure to toxicants at the cellular level in both marine and fresh water species (Gary Ostrander, 1996). As an indicator of exposure to contaminants, histology constituents a useful tool for assessing the degree of pollution, particularly for sub lethal and chronic effects (Bernet, *et al.*, 1999).

Histopathological biomarkers are closely related to other biomarkers of stress since many pollutants have to undergo metabolic activation in order to be able to provoke cellular change in the affected organism. Histopathological lesions are related to the biochemical changes that occur in the organism. As well as from chemical insult, histopathological lesions may arise from infectious diseases and parasites, provoking necrotic and degenerative alterations to which the organism responds with an inflammatory, defensive reaction (Velkova- Jordanoska, 2002; Roganovic- Zafirova *et al.*, 2003). The exposure of fish to chemical contaminants is likely to induce a number of lesions in different organs (Bucke *et al.*, 1996). Gills (Poleksic *et al.*, 1994), Kidney (Bucher and Hofer, 1993), and liver (ICES, 1997) are suitable organs for histological examination in order to determine the effect of pollution.

Teleost liver is the primary organ for biotransformation of organic xenobiotics, and probably also for the excretion of harmful trace metals, food digestion and storage and metabolism of sex hormones (Health, 1995; Hinton *et al.*, 2001). There have been numerous reports of histo-cytopathological changes in livers of fish exposed to a wide range of organic compounds and heavy metals (Hinton and Lauren, 1990; Hinton *et al.*, 1992; Hinton, 1994; Vandenberghe, 1996; Global Tox, 1997; Braunbeck, 1998). Livers of fish are sensitive to environmental

contaminants because many contaminants tend to accumulate in the liver, making this organ exposed to a much higher levels (Several orders of magnitude) than in the environment, or in other organs (Health, 1995).

The survey data of the extensive survey carried out in Canada, Europe and Australia generally show a good correlation between the concentrations of various persistent chlorinated hydrocarbons and metals and liver lesions (Malins *et al.*, 1988: Kohler, 1990; Myers *et al.*, 1992). Hinton (1994) recommended the use of neoplasms as well as megalocytic hepatosis (MH), neoplasms, foci of cellular alteration (FCA), Hydropic vacuolation (HV), hepatocellular necrosis and hyperplasia of regeneration as hepatic histopathologic biomarkers for assessing chronic toxicity in fishes. The efficacy of histological lesions as sensitive and consistent indicators of the health of wild fish populations has been demonstrated in several European and North American studies (Kohler, 1991; Kohler *et al.*, 1992; Bucke and Feist, 1993; Vethaak and Wester, 1996; Bogovski *et al.*, 1999.).

According to Johnson *et al.*, (1993) and Myers *et al.*, (1993) liver histopathological lesions are not specific to pollutants and not all hepatic lesions identified in some fishes can be used as biomarkers since certain liver lesions appear to be species specific. It has been shown that liver neoplasms were rare in young wild fish, and the risk of hepatic disease increased with fish age (Myers *et al.*, 1992; Johnson *et al.*, 1993; Myers *et al.*, 1998 a,b) presumably attributable to a longer period of exposure. Sex specific difference in cellular stress responses were repeatedly demonstrated in hepatocytes of European flounder, which may explain differences in the susceptibility of fish to toxic and carcinogenic compounds in polluted environment (Winzer *et al.*, 2001, 2002a, b).

Several laboratory and field studies by Varanasi *et al.*, (1987); Stein *et al.*, (1990); Stein *et al.*, (1992); Moore and Myers (1994); Vethaak and Jol (1996) and Vethaak *et al.*,(1996), have demonstrated contributory links between exposure to xenobiotics and the development of toxicopathic hepatic lesions. An increased number of macrophagic aggregates can be found in the liver, kidney and spleen in fish exposed to chemical pollutants, bacteria, fungi or parasites (Roganovic – Zafirova and Jordanova, 1998).

Gill histopathological changes are, in general, responsive but nonspecific to pollutant exposure. Epithelial hyperplasia with lamellar fusion, epithelial hypertrophy, telangiectasia, edema with epithelial lifting and epithelial desquamation are typical histopathological lesions of gills in response to a wide range contaminants, including organochlorins, petroleum compounds, organophosphates, carbamates, herbicides and heavy metals (Baker, 1969; Gardner and Yevich, 1970; Van der Putte and Paert, 1982 ; Hemalatha and Banerjee, 1997; Global Tox, 1997).

The reviews of Mallat (1985) and Wood (2001) have provided comprehensive information on structural changes in fish gills in response to toxicants exposure. Fish gill is a multifunctional organ responsible for respiration, osmoregulation acid-base balance and nitrogenous waste excretion. The gills are in direct contact with the contaminated medium (water) and have the thinnest epithelium of all the organs and metals can penetrate through the thin epithelia cells (Bebianno *et al.*, 2004).

The gills, which participate in many important functions in fish, such as respiration, osmoregulation and excretion, remain in close contact with the external environment, and particularly sensitive to changes in the quality of water, are considered the primary target of the contaminants (Poleksic and Mitrovic-Tutundzic, 1994; Mazon *et al.*, 2002; Fernandes and Mazon, 2003). Gills are generally considered good indicators of water quality, being used as models for studies of environment impact, such as of xenobiotics (Fanta *et al.*, 2003) being models for environmental impact assessment (Mallat, 1985: Laurent and Perry, 1991).

Changes in environmental parameters often damage the gills because of its delicate structure. Dutta *et al.*, (1996) and Wendalaar Bonga, (1997) demonstrated the effect of ambient toxicants on fish gills. Hemalatha and Banerjee (1997a, 1997b) have studied the toxic impact of the trace element Zinc (ZnCl2) on the gills and accessory respiratory organs of *Heteropneustes fossilis*.

Studies of Baker (1969); Dutta (1997); Hemalatha and Banerjee, (1997a, b) and Parashar and Banerjee (2002) reported that the significant increase in the

density of its mucous cells is the immediate morpho-pathological response of the gill of fish exposed to xenobiotics including metal salts. Heuvel *et al.*, (2000) observed mucous cell proliferation of yellow perch from oil sands reclaimed environments following longer residency periods, suggesting that this type of response may be long-term adaptation.

Kidneys of marine fish play an important role in maintaining osmotic homeostasis. Moreover, renal tissues receive large volumes of blood flow and serve as a major route of excretion for metabolites of various xenobiotics. Since renal tissues are potentially continuously exposed to toxic chemicals, the risk of effects is high. Non-specific kidney histopathological lesions (eg. degenerative changes in tubular epithelium, dilation of tubular lamina, proteinaceous or cellular casts within tubular lamina, tubular necrosis and / or epithelial desquamation, and necrosis of intestinal hematopoietic tissues) have been observed following exposure of fish to organo chlorins, petroleum compounds, organophosphate, herbicides and heavy metals (Meyers and Hendricks, 1985 ; Global Tox, 1997.)

According to Myers *et al.*, (1993) fish age, but not sex was significant in affecting necrotic and sclerotic kidney lesions and prevalence of kidney lesions in fish species was associated with xenobiotoc exposure, however, their kidney lesions were far less frequent than hepatic lesions. Owing to the poor sensitivity and lack of clear dose-response relationship kidney histopathology in fish is less commonly used as a bioindicator of xenobiotics exposure, as compared with liver histopathology.

Coutinho and Gokhale (2000) studied the histopathology of carp (*Cyprinus carpio*) and tilapia (*Oreochromis mossambicus*) subjected to in situ tests at a sewage treatment plant in India. Giensy *et al.*, (2003) made similar studies using a goldfish (*Carassius auratus*) in the USA. In situ tests, in which healthy animals are taken to the field and exposed directly to the potentially contaminated environment, have frequently been used in environmental studies (Stien *et al.*, 1998; Pacheco and Santos, 1999; Parrot *et al.*, 2000; Olsen *et al.*, 2001; Pyle *et al.*, 2001; Camargo and Martinez, 2006). Histopathology of gills, kidney and liver of a neotrophical fish caged in an urban stream studied by Martina *et al* (2007).

Histological changes associated with heavy metals in fish have been studied by many authors (Thophon *et al.*, 2003; Mohammed and Gad, 2005; Athikesavan *et al.*, 2006; Giari *et al.*, 2007., Triebskorn *et al.*, 2007; Van Dyk *et al.*, 2007).

Atif *et al.*, (2009) studied the bioaccumulation of heavy metals and histopathological alterations in liver of *Oreochromis niloticus* in relation to water quality at different localities along the river Nile in Egypt. Marchand *et al.*, (2009) studied the histopathological alterations in the liver of the Sharptooth Catfish *Clarias gariepinus* from polluted systems in South Africa.

(Atef Al-Attar, 2007) studied the influence of nickel exposure on gill structure in the teleost fish, *Oreochromis niloticus* and observed higher occurrence of histopathological lesions such as hypertrophy, hyperplasia, shortening of secondary lamellae and fusion of adjacent lamellae and even of adjacent filaments. Similar changes in gill observed by Hemalatha and Banerjee (1997) in the air breathing catfish, *Heteropneustes fossilis* intoxicated with zinc; De Boeck *et al.*, (2001) in the spiny dog fish, *Squalus acanthias* poisoned with silver; Mazon *et al* (2002) in *Prochilodus scrofa* exposed to copper; Gupta and Dua (2002) in mercury administered the air breathing fresh water *Chana punctatus*; Thophon *et al.*, (2003), Pane *et al.*, (2004) in rainbow trout, *Oncorhynchus mykiss*, subjected to Nickel; and Rangsayatron *et al.*, (2004) with cadmium.

Handy (2002) studied the effect of acute exposure to dietary cadmium and copper on organ toxicant concentrations in rainbow trout, *Oncorhynchus mykiss*. Carl Haux and Ake Larsson (2002) evaluated the long-term sub lethal physiological effects on rainbow trout, *Salmo gairdneri*, during exposure to cadmium and after subsequent recovery. Yacoub and Abdel-Satar (2003) studied the effect of heavy metals on some fishes inhabiting Bardawil lagoon. They observed degeneration and vacuole necrosis in hepatocytes, with, hemolysis and hemosiderin pigments in liver tissues.

Histopathological alterations of white seabass, *Lates calcarifer*, in acute and subchronic cadmium exposure was studied by Thophon *et al.*, (2003). Donald Versteeg and John Giesy (2004) studied the histological and biochemical effects of cadmium exposure in the blue gill sunfish (*Lepomis macrochirus*). Velkova-Jordanoska and Goce

Kostoski (2005) studied the histological analysis of liver in fish (*Barbus Meridionalis petenyi*) in reservoir Trebenista. Morphological and functional alterations induced in trout intestine by dietary cadmium and lead studied by Crespo *et al.*, (2006) Effects of sub lethal waterborne cadmium on gills in three teleosteans species: scanning electron microscopic study was conducted by Lucrecia Ferrari (2009). Nicula Marioara *et al.*, (2009) observed various tissue alterations induced by chronic cadmium intoxication in silver crucian carp *Carassius auratus*.

Roncero *et al.*, (1990) identified the acute lesions caused by experimental lead nitrate poisoning in gill of fish *Tinca tinca*. The data concerning lead toxicity are mainly related to studies with mammalian subjects and to air borne pollutants. Parashar and Banerjee (1999a, 2002) investigated the toxic impact of lead salt and lead nitrate on the accessory respiratory system of air breathing respiratory system of air breathing teleost. Toxicopathological impact of sub-lethal concentration of lead nitrate on the aerial respiratory organs of 'Murrel' *Channa striata* has been studied by (Devi and Banerjee, 2007). Adeyemo (2008) observed the gill lesions like epithelial hyperplasa, atrophy, fusion of gill filaments, degeneration and necrosis on the gill of *Clarias gariepinus* while he studied the effect of environmental relevant lead concentrations on the gill. Singhadach *et al.*, (2009) pointed out the effect of calcium pre-exposure on reducing the histopathological alterations in Nile tilapia after lead exposure. The gills were observed oedema, lamellar hyperplasia, epithelial lifting, lamellar fusion and aneurysm in the lead exposed fishes while the fishes with pre exposed calcium showed slightly alteration when compare with lead treatment groups.

The present study attempt to reveal the normal haematological parameters and assess the toxicity of sub lethal concentration of cadmium and lead on *Etroplus maculates* by investigating the haematological, enzymatical and histopathological alterations.

Chapter 3

Normal haematological variations in healthy *Etroplus maculatus*

NORMAL VARIATIONS IN HAEMATOLOGICAL PARAMETERS IN HEALTHY *ETROPLUS MACULATUS* (BLOCH)

3.1 INTRODUCTION

Despite advances in fish medicine in recent years, interpretation of fish hematology often is hampered by a lack of meaningful reference values and the bewildering diversity of fish species. Haematological studies on fishes have assumed greater significance due to the increasing emphasis on pisciculture and greater awareness of the pollution of natural fresh water resources in the tropics. Such studies have generally been used as an effective and sensitive index to monitor physiological and pathological changes in fishes (Iwama *et al.*, 1976; Chekrabarthy and Banerjee, 1988).

Haematological parameters are valuable tools for the monitoring of fish health (Jawad, *et al.*, 2004) detecting illness, and following the progress of disease and response to therapy. The use of hematological parameters is acquiring acceptance worldwide, as a tool in the management of fish farms and they are affected by many endogenous and exogenous factors such as water temperature, reproduction cycle and metabolic rate (Martinez *et al.*, 1994; Svoboda *et al.*, 2001; Kavadias *et al.*, 2003; Bayir, 2005). Establishing reference intervals for various haematological parameters of fish is important for evaluating the effects of various environmental changes on the health of populations in the wild. It is also carried out to either determine the systematic relationship among certain species or for the knowledge of their physiology (Pavlidis *et al.*, 2007)

Haematological data are used routinely in health care of humans and domestic animals. Haematological evaluations are also gradually becoming a routine practice for determining health status in fish (Tavares-Dias *et al.*, 2003;

Ranzaini-Paiva *et al.*, 2003; Cazenave *et al.*, 2005). The haematological profile of a fish population could indicate its physiological status and health and in this way hematology combined with other routine diagnostic methods could be used to identify and assess conditions that cause stress to the fish and, consequently , disease (Tavares-Dias and Moraes, 2004; Tavares-Dias and Moraes, 2006a; Tavares-Dias and Moraes, 2007; Pavlidis *et al.*, 2007).

The blood of fish has been studied under two aspects. It has been studied to determine the haematological picture of species in their natural environment so that values of each species could be standardized and so that factors that alter them could be verified. It has also been undertaken in fish living in captivity so that abnormalities occurring in the context of temperature variations, dissolved oxygen and other factors and in the context of disease in confined fish could be detected.

Increase in stocking densities can make fish more susceptible to stress and disease, in turn, may affect or cause severe stock loss (Schreck, 1996). The physiological stress response, although irritated as an adaptive response to destabilizing factors, can have damaging effects if prolonged. It is well established that continuous stress affects the behavior and normal development, with growth reduction (Jobling and Reinsnes, 1986), suppression of reproduction (Gerking, 1980), and an increase in susceptibility to infections, through immunodepression (Scheck and Bradford, 1990) which may cause mortality. There for, there has been a greater understanding of the need to establish reference haematological values in fish in order to assess health status and the subsequent diagnosis of disease.

Fish haematological studies provide an important tool in the evaluation of its physiological status, reflecting the relative health of the aquatic ecosystem and to evidence ecological degradation. Therefore, it is necessary to know the normal range of the blood parameters previous to use them as biomarkers (Luskova, 1995). However, only a few normal values for a small number of haematological parameters have been established for some teleosts, but these values range widely due to the lack of standardized collecting and measuring techniques.

Interpretation of fish haematological data is quite difficult due to internal and external variation. It is well known that blood sampling, laboratory techniques, seasonal variations, size and ontogeny of habitat, genetic properties, sex, population density, geographical dispersion, lack of food supply and stress, PH, water and transportation affect hematological data (Rehulka and Adamec,2004).

The haematological characteristics of a number of cultivable fish species have been studied with the aim of establishing normal values ranges, and any deviation from it may indicate a disturbance in the physiological processes (Rainza-Paiva *et al.*, (2000), O'Neal *et al.*, (2001)). Several of these studies were attempts to determine if significant variations from normal values of these parameters exist that could be attributable to some internal or external factors (Munkittrick and Leatherland, 1983; Gabriel *et al.*, 2001).

Haematological tests to provide information about the state of erythropoiesis. Previous haematological studies of nutritional effects (Rehulka. 2000), infectious diseases (Rehulka, 2002a) and pollutants (Rehulka, 2002b) brought knowledge that erythrocytes are a major and reliable indicator of various sources of stress. Erythrocytes reflect the state of the organism over a prolonged period of time.

Changes in hematological parameters due to unfavorable exogenous factors like poor water quality, overstocking etc. are indices of the ill health of cultivated fish. Regular monitoring of the hematological parameters of farmed fish can be used to enhance fish production (Adebayo *et al.*, 2007). The establishment of normal variation in haematological parameters is a prerequisite for the identification of stressful conditions. The normal values may vary throughout the year according to the varying eco-physiological conditions. So the range in normal haematological values was found out for a given weight in *Etroplus maculatus.*

'Normal values 'is a term which is to be understood clearly. The haematological values of a healthy fish at any time of the year should be normal values. So the important haematological parameters like haemogloin concentration, haematocrit value (Packed Cell Volume), RBC counts, WBC counts and

erythrocyte constants were estimated in the blood of normal healthy *Etroplus maculatus.*

Haematological studies of the Indian Orange chromide, *Etroplus maculatus* were carried out in order to establish a normal range of blood parameters which would serve as baseline data for assessment of the health status of the fish as well as reference point for future comparative studies.

3.2 MATERIALS AND METHODS

3.2.1 Collection of fish and transportation

The Cichlid fish *Etroplus maculates* were collected from rivers in the kadamakudy area in the Periyar River. The sampling period was from January 2001- February 2002. Every month nearly a total of 100 fish were captured by means of net. The collected specimens comprised males and females and none of them showed symptoms of stress and diseases Fishes were transported in aerated plastic buckets and large plastic bags.

3.2.2 Maintenance of fish in the laboratory

Fishes are maintained in the aquarium in large glass tank containing 500-750 liters of aged tap water. Water was changed on alternative days. Fishes were provided with commercially available pellet food and cooked rice. After feeding food particles are immediately removed from the tank. The fresh water used had a pH of 7.0±0.45, temperature of 26±3°C, dissolved oxygen content of 7.8 ppm and a salinity of zero ppt. During the acclimatization, and study periods the fish were observed daily for any clinical signs of diseases, including lack of appetite, increased opercular movements, or visible lesions of the skin, tail and fins. Signs of disease were not apparent during any period of observation. Fish were examined for any sign of infection or disease condition (Obiekezie, 1988) and only those fishes considered to be healthy were used for the study.

3.2.3 Collection of blood

Feeding was stopped 24 hour prior to the collection of blood. Fish collected from the tank causing least disturbance as it is proved that handling stress alters

several of the blood parameters. No anaesthetizing agent was used. Blood was collected by heart puncture or by the cardinal vein puncture. Blood was collected in small test tubes containing anticoagulant ethylene diamine tetra acetic acid, the anticoagulant.

3.2.4 Haematological methods

Standard techniques in haematology were employed for the estimation of primary haematological indices (Blaxhall and Daisly, 1973). Blood was collected from the caudal region by severing the caudal vein or from the direct heart puncture or from the jugular vein below the gill arch. Heparin and ethele diamine tetra acetic acid (EDTA) used as anticoagulants. Cyanomethemoglobin method was used for the estimation of hemoglobin (Blaxhall and Daisley, 1973; Houston 1990; Azizoglu and Cengizle, 1996). Packed Cell Volume (PCV) or hematocrit was determined as per micro centrifugation method (Amlacher, 1970, Jewel *et al.*, 1991 Wilhem Filho *et al.*, 1992). Red Blood corpuscles (RBC) count was done with Neubaur chamber as described by Sohn and Henry (1969). WBC count as done as per the method by Hunter and Bomford (1963). For morphological and morphometric studies stained smears of blood was used. Finally secondary Wintrobe indices or erythrocyte indices or haematological indices were determined according to Schreck and Moyle's method (1990).

3.2.4.1 Estimation of haemoglobin (Hb) content

Hb is determined by the Cyanomethemoglobin method. In this method all types of Hb will be converted first to methemoglobin and then to cyanmethemoglobin, which can be measured colorimetrically. 0.02 ml of blood was pipetted into 5 ml of Drabkin's reagent. It is shaken well and allowed to stand for 10 minutes. Sometimes a jelly like substance was seen in the solution formed by the ruptured cell wall of RBCs. It can be removed by centrifugation. Optimal density is measured at 540 nm in Bosch and Lob spectrophotometer against reagent blank. Using a commercial cyanmethemoglobin standard a standard graph is prepared from which the values of Hb can be read directly as g/dl.

3.2.4.2 Determination of Packed Cell Volume (PCV)

PCV was determined employing the microhaematocrit method. Heparinized, nonclotted blood was collected in unheparinized even bored capillaries. It was allowed to run ½ to ¾ lengths of capillary tube and the tubes were sealed with sealing wax on opposite sides. The tubes were then transferred to a high speed microhaematocrit centrifuge and were placed in the grooves of capillary head. They were centrifuged in the centrifuge at 12000 rpm for 5 minutes. PCV was measured directly on a microhaematocrit reader associated with the centrifuge as volume present

3.2.4.3 Red Blood Corpuscular (RBC) Count

Red blood corpuscles (RBC) count was done with a Neubaur chamber as described by Sohn and Henry, (1969).

Procedure

The blood was taken in a vial containing Ethele diamine tetra acetic acid (EDTA) as anticoagulant. Blood was drawn up to 0.5 mark in RBC pipette and immediately, the diluting fluid (Hayem's solution) was drawn up to the 101 mark (thus the dilution is 1:200). Pipette was shaken thoroughly and diluted blood was charged into the counting chamber, after discarding two drops. The solution was allowed to settle for few seconds and the number of RBCs was counted in five small squares of the RBC column under high power microscope and the number of RBCs per cubic mm was calculated.

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

3.2.4.4 White Blood Corpuscles (WBC) Count

WBCs were counted according to the method described by Donald Hunter and Bomford, (1963).

Procedure

Blood was collected in vials containing EDTA as anticoagulant. The blood was drawn up to 0.5 marks of WBC pipette and immediately diluted fluid, Turk's

solution was drawn up to 11 marks above the bulb. Solution was mixed thoroughly and was allowed to stand for 2 minutes. Solution was expelled a drop of fluid was allowed to flow under the cover slip. It was allowed to stand for 2 minutes and the WBCs were counted in the 4 corner square millimeters. The number of WBCs per cubic millimeter was calculated accordingly.

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

3.2.5 Calculation of RBC constants

Based on the results of the tests which measure total RBC, Hb and PCV several calculations have been derived which give quantitative information about the red blood corpuscles. These values are called RBC constants.

3.2.5.1 Mean Corpuscular Volume (MCV)

MCV, the mean corpuscular volume is the volume of the average cell or the average cell volume of all the RBCs.

$$\text{MCV} = \frac{\text{PCV \%}}{\text{RBC in million}} \times 10 \quad \text{expressed in } \mu 3$$

3.2.5.2 Mean Corpuscular Haemoglobin (MCH)

MCH is the amount of Hb in the average RBC or average amount of Hb per cell in all the red cells.

$$\text{MCH} = \frac{\text{Hb (g/dl)}}{\text{RBC in million}} \times 10 \quad \text{expressed in pg}$$

3.2.5.3 Mean corpuscular Haemoglobin Concentration (MCHC)

MCHC is the portion of the average RBC containing haemoglobin or the concentration in the average cell.

$$\text{MCHC} = \frac{\text{Hb (g/dl)}}{\text{PCV (\%)}} \times 100 \quad \text{Expressed in \%}$$

3.2.6 Preparation of blood smear and staining

A small drop of fresh blood was placed on a slide about 1 cm from one end. Another slide is placed at an angle of about 45 degree to the first slide and moved back to make contact with the drop of blood. When the blood spreads evenly along the line of contact the spreader is pushed rapidly along the line of slide. The smear is allowed to dry in the air.

For the morphological and morphometric examination of blood cells, the slide was stained using pappenheim method (Combined May-Gruenwalds and Giemsa stain). For this the slide should be first fixed in absolute methanol 3 to 5 minutes and allowed to air dry. Air dried smear fix in 2-3 drops of May-Gruenwald stain for 3 minutes. After this add equal quantity of distilled water for 1 minute. After this just decanted the stain, then stained with dilute Giemsa (10 ml Giemsa stain in 10 mil of distilled water) for 15-20 minutes. Flushed vigerously with distilled water and air dried. From each fish a minimum of 3 slides are made. The blood smears were used for the morphological observation of blood cells, for thrombocyte and total leukocyte count (WBC), and differential leukocyte counts

3.2.7 Morphometry of blood cells

The qualitative description of erythrocytes was adopted from Fijan (2002) and Valenzuela *et al.*, (2006 a).

The erythrocytes and leucocytes are measured by the calibration of an ocular micrometer with stage micrometer. The length and the width of the cells are measured and the nucleus/cytoplasmic (N/C ratio) is calculated

$$\text{N/C ratio} = \frac{\text{Length} \times \text{Width of the nucleus}}{\text{Length} \times \text{Width of the cytoplasm}}$$

3.2.8 Cytomorphology and Cytometry

Cytomorphology and staining property of different types of blood cells were studied from stained blood films using oil-immersion microscopy. The relative abundance of each leucocyte type was quantified by making a different count. Color

photographs of all cell types were taken using X 18 eyepiece and oil immersion objective.

Linear dimensions of all cell types were measured using ocular micrometer and under X 8 eyepiece and oil-immersion objective. Twenty five cells, randomly selected from different smears prepared from different fishes were measured. Long diameters (= length) of cell or cytosome and nucleus and short diameters (= width) of cytosome and nucleus. Following ratios were calculated from the data collected.

1. L / W ratio of cytosome = $\frac{\text{Length of cytosome}}{\text{Width of cytosome}}$

2. L / W ratio of nucleus = $\frac{\text{Length of nucleus}}{\text{Width of nucleus}}$

3. Ln / Lc ratio = $\frac{\text{Length of nucleus}}{\text{Length of cytosome}}$

4. Wn / Wc ratio = $\frac{\text{Width of nucleus}}{\text{Width of cytosome}}$

5. N/C ratio (Nuclear-cytoplasmic ratio) = $\frac{\text{L X W of Nucleus}}{\text{L X W of cytosome}}$

3.2.9 Statistical analysis

Haematological data were analysed with one-way analysis of variance using SPSS for Windows.

Regression analyses were employed between the various parameters measured. The coefficient of regression (r) was checked for statistical significance by the student t-test at 0.05 level of significance (Zar, 1984).

All values are presented as mean +- standard deviation. Data are presented in the form of tables, histograms and line graphs.

3.3 RESULTS

3.3.1 Morphology of RBC

Fish RBCs are nucleated. Erythrocytes in circulating blood were oval with a homogenous light pink cytoplasm. Their nucleus color was purple violet to purplish blue color and the nucleuses were centrally located. In *Etroplus maculates* most of the RBCs were elliptical in shape. Oblong cells also seen. Circular mature RBCs were also seen. But immature erythrocytes were circular in shape and they sometimes appeared in the blood smears. They were usually polychromatophilic cells smaller in size than mature erythrocytes (length 8.82 μm, width 7.83 μm). Enucleated RBCs known as erythroplastids occurred at times especially during the breeding season. They resembled mature erythrocytes except for their enucleated condition and smaller size (length 7.24μ; width 7.84 μm). The mature RBC varied in length from 9.2 μm to 13.74 μm and in width from 7.56 μm to 10 μm.

In Giemsa stained smears the cytoplasm was homogenous in appearance. Small chromatin clumps were uniformly distributed in the nucleus. No nucleolus was observed. Presence of erythrocytes with anomalous shapes multinuclear, horse-shoe shaped, kidney shaped and pointed cells were not uncommon.

Immature erythrocytes, slightly smaller than mature ones, are nearly round cells with large, round peripherally shifted nuclei. The size of the cell and the nucleus of immature erythrocytes are 10.47 X 8.34 μm and 4.9 X 4.16 μm respectively.

The cytoplasm of the immature erythrocyte is moderately basophilic and it stains grayish–blue. The peripherally shifted nucleus stains dark blue purple/ blue. The chromatin is usually clumped into a cart-wheel configuration.

In addition to the mature and immature forms, occasionally three more types of erythrocytes were noticed in the peripheral blood: microcytes, macrocytes and effete cells.

Microcytes are smaller than the mature erythrocytes and spherical in shape. Macrocytes are much bigger than the normal cells. They are usually round, or occasionally slightly irregular cells with very pale pink cytoplasm and peripherally shifted, small, nearly round, light purple or pink, diffused nuclei usually with rugged margin.

In the present study, erythrocytes cells with double nuclei (Symplasts) could see. We found erythrocytes at initial stage of a typical mitotic division that is not a characteristic process in these cells. We suppose that it possibly concerns including additional compensatory mechanism for increasing the number of erythrocytes in an environment.

Different types of Erythrocytes identified in Etroplus maculatus

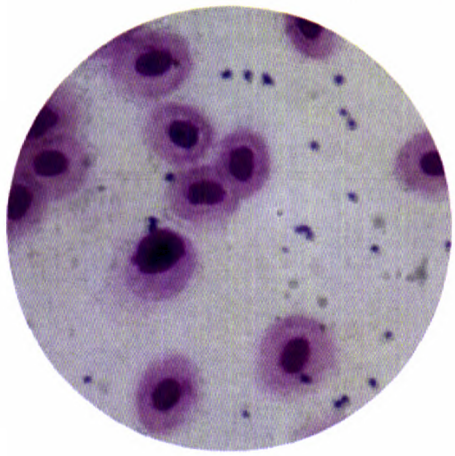


Fig: 3.1: Erythrocytes (40x)

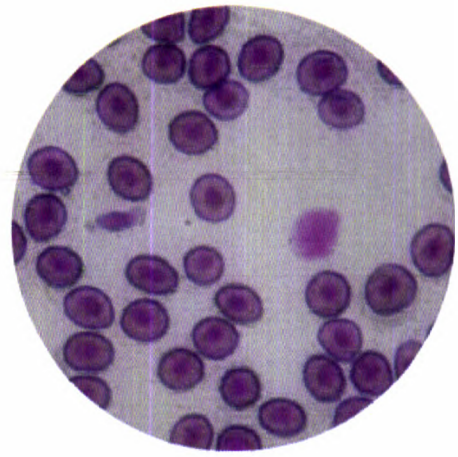


Fig: 3.2: Round Erythrocytes (Microcytes) (40x)

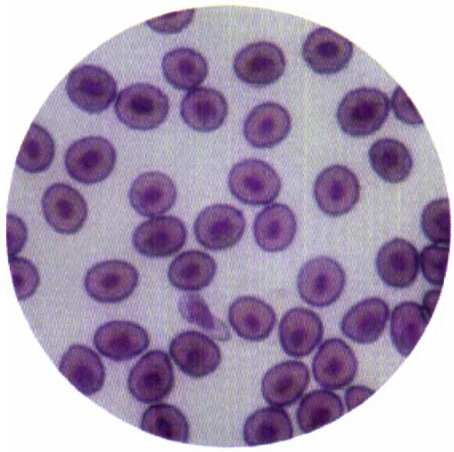


Fig: 3.3: Erythrocytes (40x)

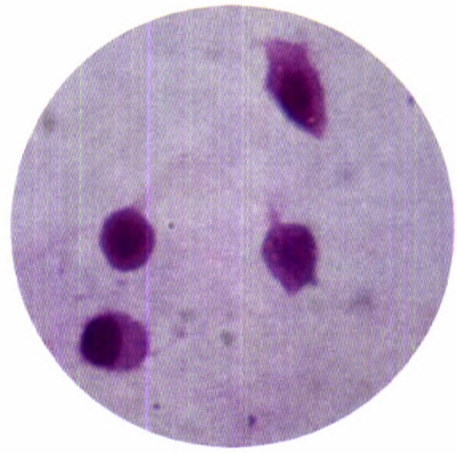


Fig: 3.4: Erythrocytes (40x)

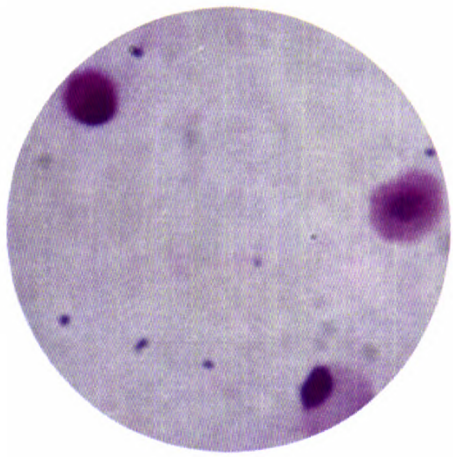


Fig: 3.5: Erythrocytes Nucleus in the side (40x)

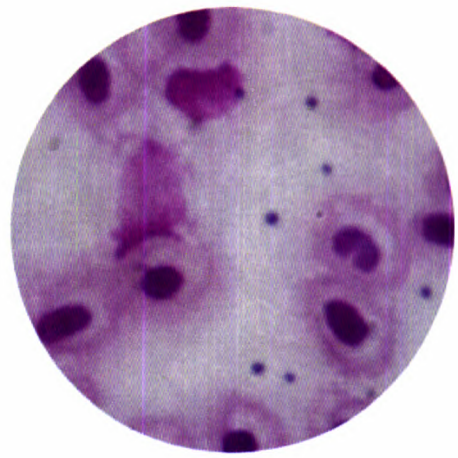


Fig: 3.6: Dividing Nucleus of Erythrocytes(40x)

The fully formed erythrocyte of the fish species were elliptical discoidal elements with centrally located nucleus. Erythrocytes in fresh blood preparations were often teardrop or triangular in outline due to a pointed pole or a slight tail at one pole. The erythrocytes examined were practically all mature. Polychromatophilic erythrocytes and erythroblasts were rarely observed in the general circulation. The cytoplasm of the fully mature erythrocytes stained pale pink, and the nuclei stained magenta with darker staining chromatin clumps; the cytoplasm of the immature cells stained grayish blue and the nuclei stained light blue with dark blue chromatin clumps.

Table 3.1

Morphometry of the erythrocytes of *Etroplus maculatus*

Erythrocyte measurements	**Mature cell (200 nos)**	**Polychromatophilic cell (100 Nos)**	**Erythroplastid (20. Nos)**
Cytoplasm			
Length (L) µm			
Mean ± SD	11.5±1.08	8.82 ±0.31	7.24± 0.36
Range	9.2 – 13.74	7.53 – 8.9	6.92 – 7.50
Width (W) µm			
Mean ± SD	7.56±1.53	7.83±0.19	7.84±0.32
Range	3.75 – 10.00	7.90 – 8.65	7.35 – 7.9
L x W	86.94	69.06	56.76
Nucleus			
Length (L)µ			
Mean ± SD	2.90±0.43	3.65±0.26	
Range	3.55 – 5.05	3.25 – 4.21	
Width (W) µ m			
Mean ± SD	2.14±0.52	3.80±0.25	
Range	1.75 – 2.67	4.05 – 4.43	
L x W	6.206	13.87	
N/C Ratio (L x W of nucleus/ Lx W of cytoplasm)	0.07	0.20	

3.3.2 Variation in normal values

The haematological profile of *Etroplus maculatus* under normal conditions: erythrocyte counts, haemoglobin concentration, haematocrit, MCV, MCH and MCHC are presented in the **Table 2.**

Table 3.2

Normal hematological values in *Etroplus maculatus*

	RBC million	**Hb g/dl**	**PVC %**	**MCVμ3**	**MCH pg**	**MCHC%**
Male (N.20)						
Mean	3.25	9.35	24.60	103.34	36.06	34.91
± SD	62.80	1.3393	1.1485	13.6406	8.1469	6.5150
Range	2.28-4.25	7.2 - 12.5	20.5 – 27.6	44.26	29.30	25.59
Female(N.20)						
	RBC million	**Hb g/dl**	**PVC %**	**MCVμ3**	**MCH pg**	**MCHC%**
Mean	3.22	10.3	25.6	89.89	39.76	43.29
± SD	73	2.4801	2.6897	28.6292	17.1315	14.3732
Range	2.22 - 4.63	6.7 – 13.5	19.7- 29.7	103.44	58.90	48.70

3.3.2.1 RBC count

In *Etroplus maculatus* erythrocyte counts were determined for 100 fishes ranging in weight from 3.9 gms to 9.7 gms (62 males 38 females) all over the year for determining the normal value for a particular size groups. RBC count varied from 228.00 to 450.00 X 10 4/mm3 in the male and 222.00 to 493.00 X 104/mm3 in the female. The mean values were found to be 325.82 ± 62.8 X 10 4/mm3 for the male and 324 73.85 X 10 4/mm3 for the female. In both sexes the lowest numbers of erythrocytes were found when the sexes were immature and temperature was low. The highest values were found while the gonads were maturing when the temperature was the highest in summer months. In the immature fish, sexual differences in erythrocyte number was not found..

3.3.2.2 Hb concentration

Hb concentration in *Etroplus maculatus* varied from 7.20 to 12. 50 g/dl in the male and 7.7 g/dl to 13.50d/l in the female. Just like RBC, maximum Hb was found during the breeding season and the minimum when the gonads were immature. No great differences in mean Hb were found between sexes.

3.3.2.3 Haematocrit (PCV)

Variation in normal values

In *Etroplus maculatus* PCV varied from 32 % to 47.51% in the male (33 numbers) and 32.50 to 53.33 % in the female (32 numbers). The mean PCV was 39.66 ±4.17 % in the male and 39.89 ±3.52 % in the female. Minimum PCV was found during December–January months and maximum from February to April months. No differences were found in mean values between the two sexes.

There is a high positive correlation between haematocrit and both erythrocyte counts and haemoglobin contents respectively in all sizes of Etroplus maculates, with mean r-values of 0.860 and 0.843 ($p < 0.05$) for Hct/EC and Hct/ Hb4 respectively (Tables 2-4)

Table 3.3

Descriptive Statistics for Normal haematological parameters of *Etroplus maculatus*

Experiment	Mean	Minimum	Range	SD	
Hb	8.19	6.71	9.89	3.18	0.6298
RBC	2.13	1.29	2.92	1.63	0.4468
PCV	23.61	18.10	28.70	10.60	2.1661
MCHC	35.09	27.17	49.94	22.77	4.8401
MCH	39.56	26.78	63.10	36.32	8.5665
MCV	116.11	80.41	182.17	101.76	27.8516
Total	37.45	1.29	182.17	180.88	39.5549

Table 3.4

Analysis of Variance for Normal haematological parameters of *Etroplus maculatus*

Source	Sum of Squares	df	Mean Square	F	p-value
Experiment	339702.390	5	67940.478	464.386	0.000
Error	34234.642	234	146.302		
Total	373937.032	239			

It has been seen that the p-value corresponding to experiment is less than the significance value 0.05; we conclude that the experiment is statistically significant.

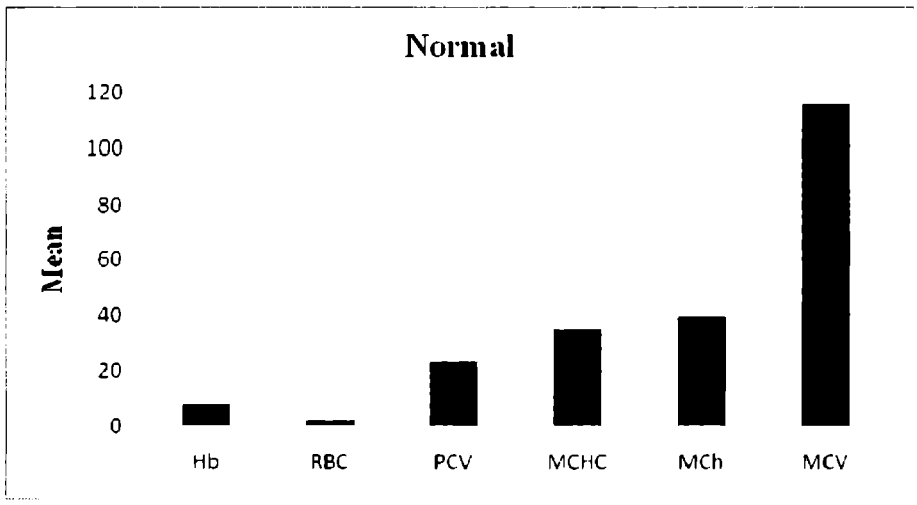


Fig: 3.7: **Normal haematological parameters of *Etroplus maculatus***

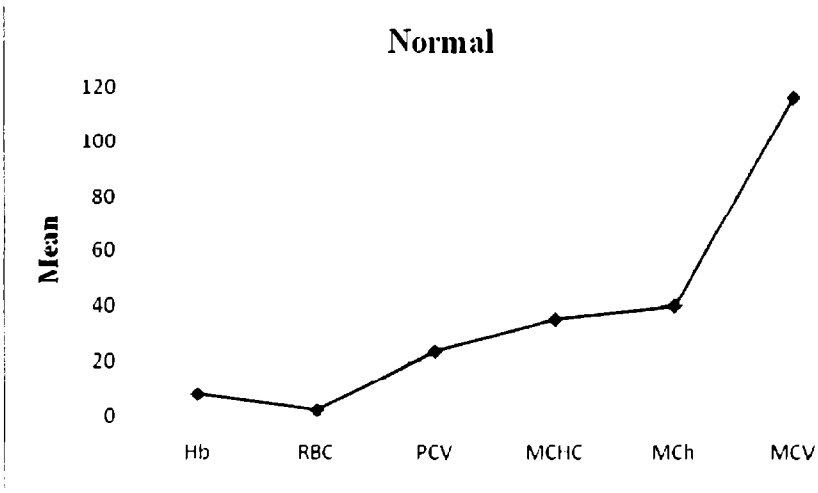


Fig: 3.8: **Normal haematological parameters of *Etroplus maculatus***

Table 3.5

Descriptive Statistics for Haematological parameters of male and female *Etroplus maculatus*

Experiment	Gender	Mean	Minimum	Maximum	Range	SD
Hb	Male	8.21	6.32	11.23	4.91	1.3393
	Female	9.90	6.01	13.50	7.49	2.4801
	Total	9.06	6.01	13.50	7.49	2.1466
PCV	Male	23.60	21.40	25.30	3.90	1.1485
	Female	23.04	18.10	25.53	7.43	2.6897
	Total	23.32	18.10	25.53	7.43	2.0613
RBC	Male	2.32	1.90	2.91	1.01	0.2922
	Female	2.64	1.62	3.23	1.61	0.5535
	Total	2.48	1.62	3.23	1.61	0.4658
MCV	Male	103.34	78.37	122.63	44.26	13.6406
	Female	89.89	43.72	147.16	103.44	28.6292
	Total	96.62	43.72	147.16	103.44	23.1595
MCH	Male	36.06	24.18	53.48	29.30	8.1469
	Female	39.76	18.61	77.51	58.90	17.1315
	Total	37.91	18.61	77.51	58.90	13.3727
MCHC	Male	34.91	26.89	52.48	25.59	6.5150
	Female	43.29	25.13	73.83	48.70	14.3732
	Total	39.10	25.13	73.83	48.70	11.8047
Total	Male	34.74	1.90	122.63	120.73	33.9811
	Female	34.75	1.62	147.16	145.54	32.2569
	Total	34.75	1.62	147.16	145.54	33.0608

Table3.6

Analysis of Variance for haematological parameters of male and female *Etroplus maculatus.*

Source	Sum of Squares	df	Mean Square	F	p-value
Experiment	227550.541	5	45510.108	314.833	0.000
Gender	0.011	1	0.011	0.000	0.993
Error	33680.881	233	144.553		
Total	261231.433	239			

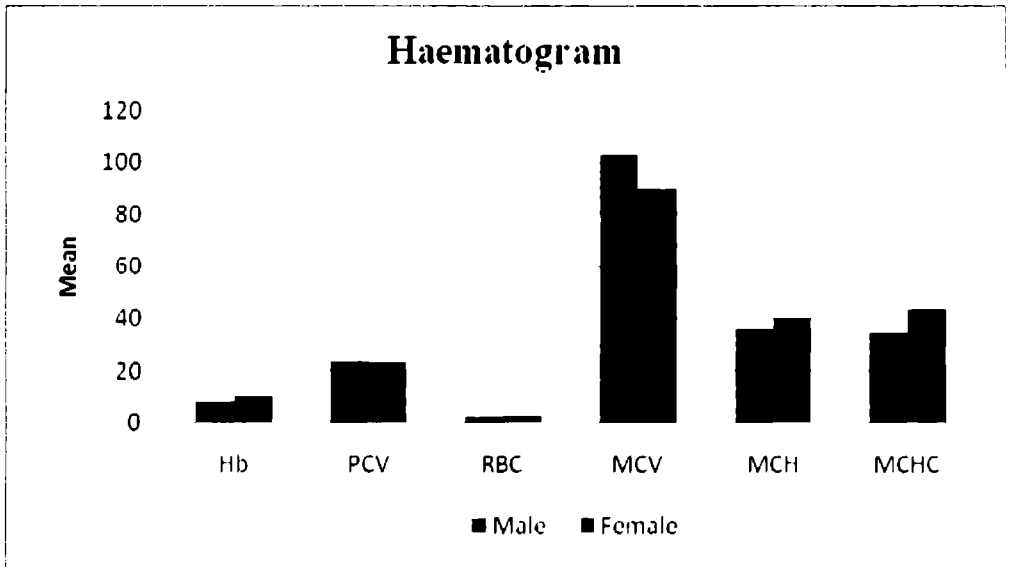


Fig: 3.9: **Haematological parameters of male and female *Etroplus maculatus.***

It has been seen that the p-value corresponding to experiment is less than the significance value 0.05; we conclude that the experiment is statistically significant. However, it is noted that the p-value corresponding to gender is greater than the significance value 0.05; we conclude that gender is not statistically significant.

3.4 DISCUSSION

The study of fish blood and fish blood cells is not a new scientific discipline; this discipline is undoubtedly nearly a century and a half old. The significance of fish haematology in disease aetiology of fishes can not be over emphasized (Kori-Siakpere and Egor, 1997).

Nutritional deficiencies, parasitic diseases, infectious diseases or organic and glandular disorders can result in situation known as dyshaemopoitic anaemia brought about by depression in erythrogenesis according to Kelly (1979). In fishes where there is reduction below the normal range of value of the erythrocyte numbers, there is correspondent reduction in haemoglobin values per cell (Larsson *et al.*, 1984).

The results presented in table show wide ranges in the values of the haematological parameters of *Etroplus maculatus* tested, with these ranges similar to those reported for other freshwater teleost species. The variability in the values of the haematological characteristics obtained in this study is similar to those reported in other studies on catfishes, such as *Clarias isheriensis* (Kori–Siakpere, 1985), four catfish species raised in a fresh water pond (Erondu *et al.*, 1993), *Heteropneustes fossilis* (Fagbenro *et al.*, 1993) and *Heterotis niloticus* (Fagbenro *et al.*, 2000). The main factors found to be responsible for the variations in the haematological make up within a given species are age, size of the fish, season of the year, nutritional status (McCarthy, *et al.*, 1973), environment (Giles *et al.*, 1984; Goel *et al.*, 1985), and infectious diseases (Amednd and Smith, 1975).

The blood of a healthy vertebrate shows correlation between the number of erythrocytes, haematocrit value and haemoglobin content (Badawi and Said, 1971). The same correlation was also observed in the values of haematological parameters of tested fish.

As pointed out by Teixeira *et al.*, (2000) reference values determined for haemogram elements may not represent precisely those of a certain population or animal species and should, therefore, be carefully interpreted, once there is a wide range of physiological variation and this variations are influenced by environmental conditions, gender, age, origin, breeding system, feeding and lineage, which may also interfere with the results obtained in tests.

3.4.1 Morphology of erythrocytes

Study of vertebrate blood cells including those of fishes, particularly teleosts, attracted scientific attention as early as 1845 when Gulliver made a detailed study of the red blood cells in scorpion fishes. Malassez (1872) proposed an inverse relationship between total count ad sizes of erythrocytes; he introduced the concept of the correlation between the activity of fish and the size of erythrocyte. Bizzazero and Torre (1883) attempted a comparative study of the blood corpuscles of different classes of vertebrates. Detailed study of teleost blood cells, their identification, classification and nomenclature have also attracted during the first four decades of this century.

It is reported that erythrocytes of fishes are usually elliptical in their form (Gulliver, 1875). But it did vary from elliptical to oblong to circular in *Etroplus maculatus*. A few oval, elliptical and oblong cells were also found. Srivastava (1968a) and Pandey *et al.*, (1976) found elliptical and circular cells in *Heteropneustes fossilis*. Smith *et al.*, (1952) also observed that the nuclei of 10 species of teleosts take the same shape as the cell in which they are found. According to Nilza de Lucas *et al.*, (2003) the erythrocytes in circulating blood in Nile Tilapia were oval with a homogenous light red cytoplasm. Their nucleus color was purple and centrally located.

The mean values for the size of the mature erythrocytes were observed to vary greatly between species of fishes (Barron *et al.*, 1956; Haws and Goodnight, 1961; Pradhan, 1961; McKnight, 1966).

Milne-Edwards (1857), Dawson (1933), and Haws and Goodnight (1961) proposed that the activity of the animal and the size of the blood cells are closely related, i.e. the more active species have smaller erythrocytes and sluggish ones have larger corpuscles. The size of the RBC reported for *Etroplus maculatus* are medium and this fish is not very active and they are not extremely sluggish.

Polychromatophilic erythrocytes in the blood has been reported by Pandey and Pandey (1977) in *Rita rita*, Boomker (1980) in *Clarias gariepinus* (1980) and

Joshi (1987) in *Clarias batrachus*, *Heteropneustes fossilis* and some other cat fishes.

Erythroplastids, the erythrocytes without nucleus was found in the blood of *Etroplus maculatus*. They were earlier reported by Lucas and Jamroz (1961) in birds and Boomker (1980) in *C. gariepinus* and *Sarotherodon mossambicus*. In the fishes under investigation they were found during the breeding season though the reason is not known. Boomker (1980) represented them as ending the line of erythrocyte development stages. Microcytes and macrocytes were at times seen in the blood of *Etroplus maculatus*. They were also reported by Pandey and Pandey (1977) in *Rita rita*.

3.4.2 RBC COUNT

Erythrocytes count at range 2.28- 4.25 X10 6 in *Etroplus maculatus*. These range within the range described by Gabriel *et al.*, (2004) (2.3 -4.3 X 106 / mm3) and Adeyemo *et al.*, (2003) (2.76 X 10 6 /mm3) and a similar observation was noted with another species such as tilapia as reported by Terry *et al.*, (2000), Adam (2004), Nilza *et al.*, (2003 and in *Clarias gariepinus* by Hassan and Hamid (2006).

Das (1965) reported that erythrocyte count tend to increase with length and age of the fish. In the present study, the erythrocyte counts of *Etroplus maculatus* were higher in the large sized specimens than in the juveniles. Preston (1960) also observed such similar findings in the Plaice, *Pleuronectes platessa*. Das (1965) reported that both the red cell number and haemoglobin concentration tend to increase with length and age.

It is noted that seasonal variations do affect RBC number (Cameron, 1970; Bridges *et al.*, 1976; Bhatt and Singh, 1986). It may be either due to the changing atmospheric temperature or by the spawning season or by both. Increases in erythrocyte number during spawning season has been reported by Ezzat *et al.*, (1973) for *Tilapia Zilli* and Fourie and Hattingh (1976) for carp. High numbers of erythrocytes are needed for the high energy demands associated with gonadial maturation. Cameron (1970) has shown that changes in RBC counts in pin fish are of some importance in meeting seasonal increase in respiratory demands. He is of

the opinion that other adjustments in erythrocyte size and in the rate of blood circulation would also be required to meet the nearly tenfold change in respiratory metabolism associated with seasonal temperature extremes. Thus the wide variation in the normal erythrocyte counts can be accounted for.

Erythrocyte count at 6.93 X 10 6 mm3 in Nile tilapia (Nilsa de Lucas, 2003) are higher than those of *Etroplus maculates*. On the other hand, it is significantly lower than those described for "*pacu*" *Piaractus mesopotamicus*, 249.4 X 10 6/mm3 (Tavares-Dias *et al.*, 1999) and Florida red tilapia *Oreochromis urolepis hornorun* X *O mossambicus*, 256.7 X 106/mm3 (Tavares-Dias *et al.*, 2001)

In fishes where there is reduction below the normal range of value of the erythrocyte numbers, there is correspondent reduction in haemoglobin values per cell (Larsson *et al.*, 1984 .According to the number of RBC, the cells become either larger or smaller in size i.e., a reciprocal relationship is said to be existed between the size and number of RBC (Malassez, 1872; Kisch, 1949; Smith *et al.*, 1952; Srivastava, 1968 a).

3.4.3 Hb concentration

Haemoglobin estimation has been widely used in fish studies, but with such a variety of methods. Blood haemoglobion concentrations (Hb) can be measured on microilitre blood samples and has been used widely with many vertebrates to provide a snapshot of general physiological condition (Frisch and Anderson, 2005; Artacho *et al.*, 2007; Banbura *et al.*, 2007; Imsland *et al.*, 2007), and an index of such things as oxygen carrying capacity (Burness *et al.*, 2000).

The haemoglobin concentration (6.87 g/dl) in *Etroplus maculatus* was similar to those reported by Hassan and Hamid (2006), Adeyemo *et al.*, (2003) and Gabriel *et al.*,(2004), but was lower than those of Red Tilapia (7.3 g/dl), Nile Tilapia (10.4 g/dl), Curimbata (7 g/dl) and hybrid Tilapia (12.7 g/dl) as reported by Adam (2004), Terry *et al.*,(2000).

The mean haemoglobin concentration value of 11.48 g/dl was reported in *Parachanna obsucra (*Adebayo *et al.*, 2007) and in *Brycon orbignyanus* Hb was in the range of 8.4 - 13.1 g/dl.

A comparative study of the blood of tropical fishes by Willmer (1934) disclosed a definite tendency for higher oxygen carrying capacity in air breathing forms. Dubale (1959) found the loading tension of oxygen to be higher in the blood of air breathers than that of water breathers. The enforced partial unsaturation of blood, resulting from the admixture of oxygenated and venous blood in its passage through the heart may be the cause of the high level of Hb occurring in the blood of many air breathing fishes (Satchell, 1976). The Hb in Clarias is much less sensitive to carbondioxide in the water (Fish, 1956). Haemoglobin concentration and haematocrit values were generally higher in males in all the months of the year than females in the fish *Silurus trigostegus* and peak values for haemoglobin and haematocrit recorded in October (Al-Hassan *et al.*,(1990).

Joshi (1980) reported higher values for the same two species of fishes. Wide variations in values of Hb for the same species might be attributed to intra-specific variations existing in fish due to different eco-physiological conditions.

Very high levels of Hb during summer months in fishes should be due the high temperature of the seasons. Another reason might be the maturity of gonads. Both these factors increase metabolic activities needing higher amounts of oxygen resulting in a rise in Hb concentration. The minimum Hb occurring during the immature period should be due to the low metabolic rate of *Etroplus maculatus* during that period. In *Etroplus maculatus*, minimum Hb was found after spawning. This should have been caused by depletion resulting from spawning.

It was reported that the lowest Hb was found with in summer in tench (Guajardo *et al.*, 2003) and mirror carp (Sahan Azizoglu, 2000). The highest and the lowest Hb concentrations were observed in January (cold season) and in September (warm season)

Haemoglobin estimation has been widely used in fish studies, but with such a variety of methods. The mean value for haemoglobin concentration of *Parachanna obscura* was 5.7 g/dl is lower than that of *Etroplus maculates*. This value falls within the range reported for other fishes like *Labeo rohita*, 6.0 g//gl for males and 6.3 g/dl for females (Seddiqui and Naseem, 1979) and *Hypophthalmichthys molitrix*, 6.11 g/dl (*Pieterse et al.*, 1981)

Das (1965) and Preston (1960) reported that haemoglobin content tend to increase with length and age of the fish. In the present study, the haemoglobin content was higher in the large sized specimens than in the juveniles.

Eisler (1965) had suggested that there was a correlation between haemoglobin concentration and activity of fish. The more active fishes tend to have high hemoglobin values than the more sedentary ones. Consequently, *Etroplus maculatus* being a relatively quiet a species has slightly lower hemoglobin concentration than the other more active species such as *Clarias buthupogen* whose mean haemoglobin concentration is high (Kori-Siakpere and Egor, 1977). The hemoglobin concentration in Nile tilapia is 10.52 g/dl, and this value is higher than that of *Etroplus maculatus*.

The studies in fish *Silurus triostegus* showed that the haemoglobin concentrations and the haematocrit value vary as the fish become older. The younger fish of age group I show relatively higher values. The value of those two haematological parameters continues to increase up to the fourth group and then decrease afterward. Thus the older fish which belong to age group V and older show comparatively lower values for each parameter (Abood, 1992).The young fish usually, show high physical activity and active feeding during growth (Chanchal, *et al.*, 1979), this will explain the high values obtained for haemoglobin concentration and haematocrit value. On the other hand, the older fish show a slow metabolic activity after a certain age (Joshi and Tandon, 1977). The lower values of haemoblin and haematocrit obtained as the age of fish increase. (Johanson, *et al.*, 1974: Hameed and Jiad, 1986: and Chaudhuri, *et al.*, 1986, Abood, 1992)

In fish blood, oxygen is carried in physical solution and also in combination with hemoglobin. So physiologically, haemoglobin is crucial to the survival of the fish as its role is directly related to the oxygen binding capacity of blood. Despite this importance *Chaenocephalus aceratus* has been reported as having no haemoglobin (Rund, 1954) indicating that in such species, oxygen is transported by blood in physical solution only.

3.4.4 Haematocrit (Hct)

Blaxhall and Daisley (1973) for instance, have reported the essence of using haematocrit to detect anaemic conditions in fishes. Several reported values for fish haematocrit fall between 20 % and 35 % (Okafor Antony, 2010) and rarely do values above 50 % been reported (Clarks *et al.*, 1976; Etim *et al.*, 1999). The mean haematocrit values for *Etroplus maculatus* of all size sizes fall within this range. That is 27.17%, 28.1 %, 28.7 and 29.2 % for juveniles and intermediates and large specimens.

Nilsa de Lucas *et al.*, (2003) the mean value for haematocrit in Nile tilapia obtained in this study is with in the range of the corresponding values described for specimens of "*piaucu*" *Lepomis macrocephalus*, *P. mesopotamicus* (Tavares-Dias *et al.*, 1999)., Florida red tilapia (Tavares-Dias *et al.*, 2000a), and *S .borelli* (Ranzani-Paiva *et al.*, 2000). This value is lower than those reported for hybrid "*tambacu* "(*Piaractus mesopotamicus* Holmberg, 1887, male X *Colossoma macropomum* Cuvier, 1818 female) (Tavares-Dias *et.al.*, 2000b) and *P.lineatus* (Ranzani-Paiva *et al.*, 2000).

Increase haematocrit have been observed in *Parachana obscura* by Kori-Siakpere *et al.*, (2005) for male fishes approaching spawning.

The mean haematocrit values in *Parachanna obscura* are almost with this 14 -28%. This is possibly due to stress of capture and transportation (Kori-Siakpere *et al.*, 2005). In *Brycon orbignyanus* the haematocrit value ranges between 35.0-47.0% (Tavares-Dias and Moraes, 2006) which is similar to those of young *Brycon amazonicus* (Tavares-Dias *et al.*, 1999) and fry of *Brycon cephalus* (Arevalo and Castellanos, 2003)

The general trend in the relationship between blood haematocrit and body length is the longer the fish, the higher the haematocrit in *Cyprinus carpio*, for example, Murachi (1959) found that haematocrit increased as the fish length increased. Similar results were obtained for *Clarius batrachus* (Joshi and Tandon, 1977).

Packed Cell Volume (PCV) is a major haematological characteristic that changes with fish activity. The reduction in PCV, in this study corroborates the finding of Jawad *et al.*,(2004) on Indian *Shad Tenualosa* and the degree of change may be due to changes in water balance which will cause increase in blood volume and in the red blood cell resulting in decreased PCV (Cameron, 1970).

In *Etroplus maculatus* highest values for PCV were obtained during the summer months when gonads were maturing and the lowest during the winter months when the temperature was low and the gonads were immature. The rate of change of metabolic activities causing changes in oxygen requirements can be the reason for this. The lowest PCV was caused by post spawning depletion of the fish.

It is generally stated that the blood haematocrit value in fish increases during the spawning season (Joshi and Tandon, 1977; Khan 1977; Leonard and McCormick, 1999). This increase has been interpreted in relation to the high-energy requirements of fish during the breeding season. On the other hand, Sano (1963) and Einszporn-Orecka (1970) reported a marked reduction in haematocrit during gonadal development in both sexes of cultured trout, interpreted as a result of the depletion of nutritive substances during spawning. Hence, two factors are probably responsible for the rise in haematocrit value: a physiological factor evoked by a high energy demand during the breeding season and an environmental factor induced by the rise in water temperature.

Several authors have shown how environmental factors such as water temperature have a direct effect on different blood parameters such as haematocrit through their effect on the haemoglobin oxygen-binding properties and thus on oxygen transport (Di Prisco and Tamburrini, 1992; Wells, 1999). Increases in osmoregulatory work might be expected to produce an increase in the blood oxygen carrying capacity, which in turn would bring about a significant change in the haematocrit value, by the same reasoning as for temperature. However, changes in water balance will cause an osmotic effect in the red blood cell, finally increasing haematocrit (Cameron, 1970).

In *Etroplus maculatus* just like erythrocytes number and hemoglobin concentration, PCV is average. High PCV values for the fishes were reported by

Joshi (1980). Just like RBC and Hb, Srivastava (1968a) reported low values for mean PCV too.

Fouri and Hattingh (1976) suggested that the differences in haematocrit between the two sexes are genetically determined, although Raizada *et al.*, (1983) considered that the differences might be due to the higher metabolic rate of males compared to females Chaudhari *et al.*, (1986) and Jawad *et al.*, (2004) suggested that an increase in fish activity with an increase in size.

The general trend in the relationship between blood haematocrit and body length is the longer the fish, the higher the haematocrit in *Cyprinus carpio*, for example, Murachi (1959) found that haematocrit increased as the fish length increased. Similar results were obtained for *Clarius batrachus* (Joshi and Tandon, 1977).

Jawad *et al.*, (2004) reported that Male *T.ilisha* showed higher blood haematocrit values than females in all the length groups studied. This is in agreement with results from other fish species *Telapia zilli*, Ezzat *et al.*, (1973); *Cyprinus carpio* Fourie and Hattingh (1976); *Cyprinion macrostomus* Al-Mehdi and Khan (1984); *Amphiprous cuchia* Banerjee (1986). Fouri and Hattingh (1976) suggested that the differences in haematocrit between the two sexes are genetically determined, although Raizada *et al.*,(1983) considered that the differences might be due to the higher metabolic rate of males compared to females. Our results support this suggestion, which has been related to an increase in fish activity with an increase in size (Chaudhuri *et al.*, 1986)

Preston (1966) equally noted increased haematocrit values for male fishes prior to the time of spawning.

During the life cycle of the fish, blood undergoes regular changes of haemoconcentration and haemodilution. These changes are mainly affected by the ecophysiological conditions of the fish (Khan, 1977 and Joshi and Randon, 1977). Chanchal *et al.*, (1979) reported that the fish, *Anabas testudeneus* shows the peak values of haematocrit value in May and October. On the other hand, Joshi and Tandon (1977) observed the peak values in May for *Heteropenuests fossilis* and *Mystus vittatus*. In the present study, the peak of both haemoglobin concentration

and haematocrit value for both sexes were in October and this coincides with the results of Chanchal *et al.*, (1979) and Raizada *et al.*, (1983). The peak in October may be suggested due to the favorable conditions of the environment and the higher metabolic activity of the fish, which lead to the more intake of food. The lowest value obtained in March and this could be due to the beginning of the maturation period. It is true that when the gonads are fully developed, fish consume very little amount of food because the major part of the body cavity is filled up by the developing gonads, resulting in low values of haemoglobin concentration and haematocrit. Lysaya (1951), Robertson *et al.*, (1961) and Hutton (1967) have reported the same observations on Salmon undergoing spawning migration. The lower food production as well as the metabolic rate of the fish slows down, resulting in low consumption of food. The higher haematological values during the summer months could be attributed to the increase in the level of adrenaline and nor adrenaline hormones due to the changes in the environment (Nakanoa and Tomlinson, 1967).

3.4.5 MCV

MCV values are important in the sense that stress makes erythrocytes to swell causing increased MCV (Holeton and Randall, 1967; Soivio *et al.*, 1974 a,b; Soivio *et al.*, 1977 and Nikinmaa, 1981). MCV values showed wide variations during different seasons of the year. The reduced MCV value was usually found during gonadial maturation as erythropoiesis happened at this time liberating small immature cells into the blood stream.

The MCV values derived in the present investigation are smaller than those presented by Srivastava (1968a) for tilapia fishes. Variation in populations or eco-physiological conditions could have caused the disparity in Values.

High MCV suggests that haemo-dilution mechanism is operational; the mean corpuscular volume gives an indication of the status or size of the red blood cells and reflects an abnormal/normal cell division during erythropoiesis. The increase in MCV may be attributed to the swelling of the erythrocytes resulting in macrocytic anaemia. Such an increase in erythrocyte size is generally considered a response against stress and would be a consequence of several factors like high

PCO2, high lactate concentration or low PO2 in the blood, leading to a low ATP concentration, which would increase the oxygen affinity of blood (Soivo and Nikinmaa, 1981). In this instance, it is difficult to ascribe the swollen red blood cells to one of these factors; the increase in MCV observed, therefore, needs further elucidation.

But MCV was found to decrease with an increase in body weight in. *Heteropneustes fossilis* ((Pandey *et al.*, 1976) and in *Sarotherodon mossambicus* (Chaudhari *et al*, 1986c) observed the phenomenon. According to Smith (1977), the relationship of MCV to W is complex and reasonably variable i.e. positive or negative correlations occur at various times of the year. To cope with the increased need of oxygen with higher weights, blood cells might have reduced their volumes. It is established that small cells having greater surface/volume ratios are most efficient in gas exchange (Hartman and Lesler, 1964). Such efficiency would be of advantage in an environment with low dissolved oxygen.

The higher value of MCV (148.8 u3) in Nile tilapia (Nilsa de Lucas *et al.*, 2003) and for '*pacu*' is 117.6 u3 and for Florida red tilapia is 113.6u3.

3.4.6 MCH and MCHC

No sex difference in MCH and MCHC were found for *Etroplus maculatus*. Just like other haematological parameters, the highest MCH and MCHC were found during the summer months. These high values are a reflection of very high Hb values during this time, as MCH and MCHC are derivatives from Hb. MCH and MCHC were found to be directly related to Hb in both sexes. The MCH and MCHC values reported by Srivastava (1968c) for *Heteropneustes fossilis* and *Clarias batrachus* and the MCHC values by Pandey *et al.*, (1976) for *H. fossilis* are not at par with the results of present investigation. The food availability, feeding regime, the nature of the habitat, the method of Hb and PCV determination all these affects the haematological values. So it is only natural that intra-specific variation may occur from region to region.

Sex doesn't seem to have any effect on the haematological parameters of *Etroplus maculatus*. In *Heteopneustes fossilis* and *Clarias batrachus* sex doesn't

seem to have any effect on the haematological parameters except for certain maturity stages during the breeding season. Several studies on Salmonoid fishes have demonstrated a sexual dimorphism in mature males and females with respect to erythrocyte count or PCV values (Snieszko, 1961; Roberstson *et al.*, 1961; Poston, 1966; Lane, 1979). But Conroy (1972) and Korzhuev *et al.*, (1982) failed to observe any sexual differences in PCV or RBC count in mature *Salmo salar.* Bhagat and Banerjee (1986) suggested that there is no distinct sexual difference in haematological parameters in *Amphipnous cuchia* and the differences occurring are strictly seasonal

Acoording to Ibrahim *et al.*, (2003) erythrocyte indexes (MCV, MCH, MCHC) and thrombocyte numbers were not differed between male and female fish. Differences in levels of some haematological parameters could be explained with effects of body size and ecological conditions.

It is apparent from the results that for *Etroplus maculatus* normal haematological parameters vary at different times of the year and the variation depends on the body weight, physiological condition of the fish, temperature and season. Sex of the fish doesn't seem to have much effect on the haematological parameters except for some stages during the breeding season. Occurrence of immature erythrocytes is a common feature of teleost fishes. Erythroplastids were usually seen during the breeding period though the reason is not unknown.

Correlation between haematocrit, haemoglobin and red cell count used in the computation of the haematological indices, have been demonstrated for several species (Eisler, 1965; Summerfelt *et al.*, 1967; Houston and Dewilde, 1968.

3.4.7 Haematological factors in relation to sex

In the assessment of the blood parameters of goldfish, *Carassius auratus*, Summerfelt (1967) observed that males consistently had significantly higher haematocrit values than the females and suggested the need to separate blood component data on the basis of sex to avoid attributing sex differences to other factors. Kori- Siakpere (1985) and Gabriel *et al.*, (2004) noted wide variations in the Hb, PCV and RBC indices of *Clarias ishierensis* from the wild. Furthermore, they

recorded variation in the values of the various blood parameters within the same sex.

The foregoing results amply show in *Etroplus maculatus*, all the haematological values were predominately higher in the male than in the respective female. It is postulated that the reason for having higher haematological values in male fish are primarily due to its being biochemically as well as nutritionally richer than the female (Joshi, 1973). Where much of the metabolites and nutrients are continuously being exhausted in the development of the ovary. During the pre spawning period males feed almost with the same intensity, whereas feeding depletes in females due to gradually decreasing space in the abdominal cavity following the rapid development of the gonads. Naturally, though the metabolic demands may be higher in females during spawning period, all the factors make the female fish poorer in various haematological parameters at the same time. A temporary microcytic anaemia may be present.

A few earlier workers like Slicher (1961) and Summerfelt *et al.*, (1967) in *Carassius*, Radzinkaya (1966) in Misgurnus, Snieszko *et al.*, (1966) in Salmo, Banerjee (1966) in Anabas and Einszporn-Orecka (1970) in Tinca and Joshi (1980) in certain fresh water teleosts in India noted higher values of PCV, TEC and HB contents in the males as compared to the females, respectively. Mulachy (1970) also noted that male Esox always had higher values of TEC, PCV and Hb contents. Besides, she also pointed out that females had narrow range variations as compared to the males. This is almost the same as has been observed in the present study. Increase haematocrit have been observed in *Parachana obscura* by Kori-Siakpere *et al.*, (2005) for male fishes approaching spawning.

Slicher (1961) and Joshi (1980) suggested that certain hormonal activity is more or less responsible for such intra species sex-related variations. This is further confirmed by the observations of Haws and Goodnight (1962) and Poston (1966), who noted sharp differences in blood values of certain sexually mature and immature fishes. But Mc knight (1966), contrary to all these above findings, never found any sex-related variation of blood values in *Prosopium williamsonii*. Apparently, such contradictory findings are scant in the existing literature. However

more detailed studies are indeed, required on a still greater number of specimens and species before making a final decision. Since, if hormones are concerned with such phenomena of physiochemical importance, then sex-linked genes or internal physio-chemical milieus of the respective sex could also be involved.

Kori-Siakpere and Egor (1997) observed differences in haematology for different sexes of *Clarias buthupogon*. According to Dacie and Lewis (1991) who believed, gender has a great influence on haematology of fish and included gender among factors influencing haematology, While Etim *et al.*, (1999) did not observe any difference between male and female of *Chrysicthys nigrodigitatus*.

The influence of sex on certain red blood components has also been reported (Hlavova 1993b; Luskova, 1995; Luskova *et al.*, 1995). However, several neotrophical species, such as *Rhamdia hillari* (Kavamoto *et al.*, 1983), *Prochilodus scrofa* (Ranzani Paiva and Godinho, 1985), *Brycon* sp. (Ranzani Paiva and Godinho, 1991) and *Prochilodus lineatus* (Parma de Croux, 1994) did not present differences between sexes. Cazanave *et al.*, (2005) could not find any differences in blood parameters of *Corydoras paleatus* between males and females when evaluated with or without consideration of the season.

Hasson *et al.*, (1990) suggested that the haemoglobin concentration and haematocrit values showed a marked variation in male and female fishes throughout the year. Hb concentration and Hct values were generally higher in males in all the months of the year than females.

Kori-Siakpere (1985) and Gabriel *et al.*, (2004) who noted wide variations in the Hb, PCV and RBC indices of *Clarias ishierensis* from the wild. Similar observations had been made in other fish species and were attributed to intrinsic factors (Burton and Murray, 1979; Etim *et al.*, (1999). Further more Gabriel *et al.*, (2004) recorded variations in the values of the various blood parameters within the same sex.

3.4.8 Haematological parameters in relation to Season

Bridges *et al.*, (1976) had demonstrated significant seasonal differences in red blood variables, occurring with relation to the reproductive activity of

Pseudopleuronectes americanus. According to Joshi (1982), the lowest values of red blood parameters were usually recorded in spent fish, or during winters when the temperature was quite low and the food scare. Preston (1960) and Mahagen *et al* (1979) observed seasonal fluctuations in haematological parameters in haematological parameters while Smith (1977) noted a correlation of MCV to weight which ranged from highly negative to highly positive based on the season. The red blood parameters of *C. paleatus* did not show significant changes associated with seasonality. Considering that red blood parameters in *C.paleatus* are not sensitive to changes in the maturation stage, sex or seasons blood constituents of *Colossoma macropomum* revealed an increase in both haemoglobin content and erythrocyte count, when fish were exposed to low oxygen concentrations (Saint-Paul, 1984). Lochmiller *et al.*, (1989) found that most of the haematological parameters measured in a population of *Morone saxatilis* were significantly increased as DO decrease.

The seasonality of haematological characteristics in fish may be related to natural physiological cycles, environmental conditions or both. Luskova *et al.*, (1995) observed variations of some parameters in *Chondrostoma nasua*, coincident with both spawning and water temperature. Cazanova (2005) hypothesized that haematological differentiation observed between fish captured at different sites could be attributed to different environmental conditions. Thus changes in haematology could indicate that fish are exposed to environmental stress. Changes in haematological parameters associated with low levels of dissolved oxygen have been mentioned in a few field studies.

Poston (1966) equally noted increased haematocrit values for male fishes prior to the time of spawning. The blood of a healthy vertebrate shows correlation between the number of erythrocytes, haematocrit value and haemoglobin content (Badawi and Said, 1971). This same correlation was also observed in the value and haemoglobin content (Badawi and Said, 1971) and (Adebayo *et al.*, 2007). PCV is positively correlated to the haemoglobin content (HB) while it is negatively correlated with erythrocyte and leucocyte numbers (P less than 0.01) The leucocytes counts in *Parachanna obscura* show positive correlation with

haemoglobin content and erythrocyte counts while it is negatively correlated with PCV.

The studies of Chaudhuri *et al.*, (1986c) and Ruparaelia *et al.*, (1986) on *Sarotherodon mossambicus* showed that total erythrocyte count, haemoglobin content and PCV varied as the fish increased in size with age. Since oxygen consumption is a function of weight (Smith, 1977), with an increase in weight, the oxygen requirements of the body will increase necessitating increased number of erythrocytes. From the present results, it is apparent that under normal conditions, Hb concentration and PCV have a positive linear relationship with RBC number. So elevation in erythrocyte number simultaneously results in increased concentration of Hb and PCV.

Houston and Wilde, 1972; Van Vuren and Hattingh,(1978) and Aysel Sahan *et al.*, (2007); reported that length and weight discrepancies of various fish species had no significant effect on haematological parameters measured.

Denton and Yousef (1975) suggested that decreases in haematological parameters within cold seasons were associated with lack of food. Therefore, low Hb, MCH and MCHC values during cold seasons were probably related to lack of food and/or adaptation to cold environment. Determination of haematological parameters of native fish is helpful in obtaining population properties and in describing properties of pollutants (Hickey, 1976). In this work, significant changes in all studied haematological parameters were observed throughout the year. The highest value in RBC, Hct, MCV and WBC were found in my (prespawning period) and summer and the lowest in cold seasons (autumn and winter). Therefore, they were very positively affected by water temperature and GSI fluctuations. These results agreed with some previous studies (Martinez *et al.*, 1994; Frangioni *et al.*, 1997; Collazos *et al.*, 1998; Guijarro *et al.*, 2003). Martinez *et al.*, (1994) suggested that there was a clear correlation among RBC, HB, Hct and environmental factors and water temperature was the most effective one. Guijarro *et al.*, (2003) reported that owing to high temperature, oxygen availability was reduced during summer in tench. It was suggested that WBC was affected by some factors such as water temperature and reproduction period (Bayir, 2005). Mevlut aras *et al.*, (2008)

reported that in Chub (*Leuciscus cephalus*) maximum RBC, Hct, MCV and WBC values were determined in the prespawning months. Hence in this investigation, the high RBC, Hct, MCV and WBC during prespawning period and warm months were explained with increasing energy needs for reproduction, and decreasing oxygen amounts.

Contrary to other haematological parameters; Hb, MCH and MCHC were quite negatively correlated with temperature changes and these parameters had no correlation with GSI.

3.5 CONCLUSION

Haematological examinations, and the correct interpretation of the results, are becoming of increasing importance in fish farming activities, and their routine utilization is highly recommended as a practical tool to "get to grips" with implementing standard diagnostic procedures in investigating problems associated with fish diseases and parasites. As haematology assessment is gradually becoming routine practice for intensively bred fish, since intensive aquaculture needs accurate information for identification and control of stress situations and/or diseases in order to ensure healthy fish, the evaluation of blood parameters may be quickest way to detect these symptoms. There for, there is an urgent need reliable normal database to be available for species of economic importance. In conclusion, the variation in methodology used for haematological studies, instant changes in physical and chemical properties of micro-environment in which fish lives makes comparison of literature and the establishment of limits for haematological data difficult. Therefore, we propose separate data collection and comparison from healthy and unhealthy fish and also between sexes to obtain further haematological data.

Chapter 4
Leucocytes and Related cells of *Etroplus maculatus*

LEUCOCYTES AND RELATED CELLS OF *ETROPLUS MACULATUS*

4.1 INTRODUCTION

Fishes form the largest group of vertebrates exhibit enormous diversity in a range of features such as morphology, physiology, habit, habitat, immune responsiveness etc. Hematology is, like wise, one aspect that shows a wide range of intra-and interspecific variations in fishes. With the increasing emphasis on pisciculture and even greater awareness of the pollution of natural water resources, haematological studies in fishes have assumed greater significance. A clinician, in human medicine or a veterinarian, knows the value of these studies for diagnostic purposes.

Studies of blood parameters have been carried out to either determine the systemic relationship among certain species or for the knowledge of their physiology (Pavlidis, *et al.*, 2007). The haematological profile of a fish population could indicate its physiological status and health and in this way haematology combined with other routine diagnostic methods could be used to identify and assess conditions that cause stress to the fish and consequently, disease (Tavares-Dias and Moraes, 2004; Tavares-Dias and Moraes, 2006a; Tavares-Dias and Moraes, 2007; Pavlidis., 2007). Red blood cell parameters can be used for the diagnosis of anaemia while the leukocytes, the primary line of immunological defense, provide an important representation of defense cells throughout the body (Tavares-Dias and Moraes., 2004; Tavares-Dias and Moraes, 2007; Affonso *et al.*, 2007; Pavlidis., 2007) Thus, one of the most elementary ways to assess the immune system is to explore changes in the number of or appearance of circulating white blood cells (WBC). However, it is generally agreed up on that fishes, like all other vertebrates, have a common leucocyte pattern consisting mainly of granulocytes,

monocytes and lymphocytes. What is still a matter of controversy is the identity and relative function of these cell types.

The differential blood cell count, as well as other haematological procedures, is important tools in the clinical laboratory to aid in the diagnosis of blood dyscrasias. Increased eosinophils counts have been noted in infections with a tendency to accumulate in and around the active pathological lesions (Jakowska, 1956). The degeneration of leucocytes and thrombocytes were observed in fish with viral diseases (Watson *et al.*, 1956). Elevated lymphocyte counts were noted when handling fish (Engel and Davis, 1964). Prolonged oxygen deficiency was found to result in lympopenia (Belova, 1966) while environmental stresses have effected increased eosinophilic counts (Gardner and Yevich, 1969) where as thermal stresses affected the abundance of lymphocytes, as well as eosinophils (Pickford, *et al.*, 1971).

Low oxygen content in the water produced increased erythrocyte count (Bonnet, 1929), but erythrocyte abundance was altered with temperature variations (DeWilde and Houston, 1967). Cellular and humoral immunity in all vertebrates are mediated by complex interactions essentially between various blood cells (Rowley *et al.*, 1988). The knowledge of the function and interrelationships of the leucocytes, the major seat of cellular immune system of vertebrates, is very essential in gaining proper insight into the immunity mechanisms. Accurate identification of the cell types undoubtedly is the stepping stone to the field of cellular immunity. Recent studies have concentrated more on the humoral aspects of fish immunology, than on cellular aspects (Hine *et al.*, 1987). This may be because of the difficulties in working with fish blood, particularly with fish leucocytes (Ellis, 1977). Leucocytes of fishes are not as highly developed and as differentiated as mammalian leucocytes; distinguishing between the cell types is undoubtedly an extremely difficult task. (Bell, 1976; Ellis, 1977; Cannon *et al.*, 1980). Further the terminology applied to fish blood cells has been borrowed from mammalian, particularly human haematology despite the lack of evidence of true analogy between fish and mammalian cells. Cellular nomenclature used in one species can only be applied to another only if the nature of these cells is in general accord with

three basic criteria: morphology, physiology and ontogeny (Ellis, 1977). Little work has so far been done on the function of most fish leucocytes. Though with the exception of the granulocytic series, there is enough known to assign a nomenclature carrying significant functional implications; albeit the nomenclature applied to certain fish leucocytes must be regarded as tentative (Ellis, 1977). Even less is known of the ontogeny of fish blood; the available evidence is primarily circumstantial.

The detailed structure of fish white blood cells and the terminology of their various types still remain controversial. There is a lack of consistency in the classification of five white blood cells and there is no uniform nomenclature either for them. The ease determination of the leukocytes type can be hampered by difficulties in classifying certain types of granulocytes. In the fish circulating blood besides mature cells there are also blastic cells and maturing cells of particular development lineages (Tavares-Dias and Moraes, 2004; Tavares-Dias and Barcellos, 2005 ; Tavares-Dias, 2006; Tavares-Dias and Moraes, 2007). Thus this inconsistency in cell nomenclature and appearance has lead to confusion regarding the identity of some cells. The most accurate classification of fish leucocyte based on conventional staining techniques is perhaps that worked out by Ivanova (1983). Her basic criteria for classification were function, individual development and morphology in the descending order of priority.

Leucocytes are chiefly involved in specific and non-specific defence mechanisms. A number of enzymes, including peroxidases, phosphatases, phosphorylases, esterases and dehydrogenases, have been found to be localized in leucocytes where they control and regulate antigen trafficking and thus assist in the defense mechanism. These enzymes have also been localized in piscine leucocytes.

In human medicine leucocyte enzyme chemistry is used as a routine method to differentiate between cell types (Hayhoe and Quaglino, 1980), to determine activation state (Cochin, 1978; Karnovsky and Lazdins, 1978; North, 1978), to differentiate between T and B lymphocytes (Catovisky and Enno, 1977), to differentiate within T cell subpopulations (Davey *et al.*, 1980; Zicca *et al.*, 1981) and to identify non-T and non-B lymphocytes (Grossi *et al.*, 1982). Cytochemical

tests help in making accurate cytological diagnosis and in demonstrating abnormalities in individual cells and certain tests are of special use in detecting and characterizing leukaemia (Dace and Lewis, 1975). "Furthermore, enzyme cytochemistry may suggest function: peroxides, lysozyme and lysosomal hydrolases are associated with death and destruction of pathogens (Neeman *et al.*, 1974; Spintznagel, 1977; Karnovsky *et al.*, 1981) and neutral proteases are associated with tissue destruction (Baggiolini *et al.*, 1980); though the function of alkaline phosphatase (Wilson *et al.*, 1983) and esterases (Luppa and Andra, 1983) are still poorly understood" (HIne *et al.*, 1987). Thus, the applications of enzyme cytochemical techniques may help in differentiating fish leucocytes and in defining the normal and or altered (as in pathological states) function. However, the primary requisite to this end is establishing the normal enzyme patterns in fish leucocytes.

Apart from being useful for identifying cell types in blood and tissue, cytochemical staining is also critical for identifying immunological cell types associated with developmental and pathological processes (Ueda *et al.*, 2001; Petrie-Hanson and Peterman, 2005). Presence of glycogen (Veiga *et al.*, 2000; Ueda *et al.*2001; Vale *et al.*, 2002; Rough *et al.*, 2005) and alkaline phosphatase (Meseguer *et al.*, 1994; Burrows *et al.*, 2001) in leukocytes may be associated with phagocytosis. This requires the consumption of energy from both endogenous and exogenous sources (Hayhoe and Quaglino, 1994; Ueda *et al.*, 2001). Peroxidase is a lysosomal enzyme. Which takes part in intracellular digestion and modulation of phagocytic activity of leukocytes (Hayhoe and Quaglino., 1994; Veiga *et al.*, 2000.; Ueda *et al.*, 2001; Vale *et al.*, 2002.; Azevedo and Lunardi, 2003). Esterases are enzymes also related to cellular defense, facilitating diapedesis, cell migration through tissue, toxic product and microorganism inactivation and tumour cell destruction (Hay hoe and Quaglino., 1994; Casaletti-Rosa and Lunardi, 1997).

Cytochemistry has proven effective in differentiating specific cell lineages and elucidating their functional properties. This study utilized a range of cytochemical techniques to further investigate the leucocyte populations from *Etroplus maculatus*. This analysis provided clear insight into the structure and

function of the leucocytes from the fish, which were found to be broadly similar to those of other fish species.

The present study carried out on the cichlid fish *Etroplus maculates* describes the occurrence of different types of leukocytes by the normal staining methods and by the cytochemical methods.

4.2 MATERIALS AND METHODS

Collection, transportation and maintenance of fish in the laboratory were same as that described in the chapter 1.

4.2.1 Collection of Blood sample

Apparently healthy fish were selected for haematological study. Blood sample was collected from the cardinal vein or directly from the heart of unanaesthetized fish.

4.2.2 Routine staining of blood smear

Fresh blood was used for the preparation of smears. The smears fixed with May-Gruenwald –Giemsa stain (MG-G).The preparation of the smear and staining procedure were same as that described in chapter 1.

4.2.3 Cytomorphology and Cytometry

Cytomorphology and staining property of different types of blood cells were studied from stained blood films using oil-immersion microscopy. The relative abundance of each leucocyte type was quantified by making a different count. Color photographs of all cell types were taken

Linear dimensions of all cell types were measured using ocular micrometer and under X 8 eyepiece and oil-immersion objective. Twenty five cells, randomly selected from different smears prepared from different fishes were measured .Long diameters (= length) of cell or cytosome and nucleus and short diameters (= width) of cytosome and nucleus. Following ratios were calculated from the data collected.

1. L / W ratio of cytosome = $\frac{\text{Length of cytosome}}{\text{Width of cytosome}}$

2. L / W ratio of nucleus = $\frac{\text{Length of nucleus}}{\text{Width of nucleus}}$

3. Ln / Lc ratio = $\frac{\text{Length of nucleus}}{\text{Length of cytosome}}$

4. Wn / Wc ratio = $\frac{\text{Width of nucleus}}{\text{Width of cytosome}}$

5. N/C ratio (Nuclear-cytoplasmic ratio) = $\frac{\text{L X W of Nucleus}}{\text{L X W of cytosome}}$

4.2.4 Enzyme cytochemistry

Fresh blood samples collected from healthy adult specimens of the *Etroplus maculatus* were taken for smear preparation in cytochemical studies. The fresh air dried smears were fixed in an appropriate fixative recommended for each test. Localization and activity of the following five enzymes in the blood cells were studied. The methods adopted were from the authors shown against each.(1) Peroxidase (PER: Kaplow, 1965), (2) alkaline phosphatase-(ALP: Cartwright, 1968), (3) Acid phosphatase- (ACP-(Brown, 1980), (4)α-naphthyl acetate esterase-ANAE (Yam *et al.*, 1971a) and Naphthol ASD chloroacetate esterase –(ASDE; Yam *et al.*, 1971a).

Enzyme activity was determined visually based on the intensity of the specific color developed to each cell. The activity was scored as follows.

Negative	-
Weak	+
Moderate	++
Strong	+++
Inconsistent	-/+

Blood smears from 100 specimens of *Etroplus maculatus* were examined during the present study. A minimum of three smears were prepared from each fish so as to have one duplicate for each staining test.

4.3 RESULTS

4.3.1 Cytomorphology, Cytometry and leukocyte differential count.

The formed elements of the circulating blood of Etroplus maculates consist of nucleated erythrocytes, thrombocytes, and leukocytes. The leukocytes mainly classified into two based on the presence of granules. That is agranulocytes and granulocytes. Agranulocytes includes lymphocytes and monocytes. Granulocytes include neutrophils, basophils and eosinophils.

4.3.2 Erythrocytes

Erythrocytes are nucleated in *Etroplus maculates* like any other fish species. Both mature and immature erythrocytes are present in the peripheral blood, immature ones being sparsely distributed. Mature erythrocytes are oval to subspherical cells with centrally placed oval or subspherical nuclei. The average size of the cell is 11.25 X 8.35 μm; the nucleus measures on an average 3.46 X 2.94 μm.

The cytoplasm of the mature stains homogeneously pale pink without any vacuoles and granules. The nucleus with densely packed chromatin, stains dark purple in fresh smears and deep blue in old preparation.

Immature erythrocytes, slightly smaller than mature ones, are nearly round cells with large, round peripherally shifted nuclei. The size of the cell and the nucleus of immature erythrocytes are 10.47 X 8.34 μm and 4.9 X 4.16 μm respectively.

The cytoplasm of the immature erythrocyte is moderately basophilic and it stains grayish–blue. The peripherally shifted nucleus stains dark blue purple/blue. The chromatin is usually clumped into a cart-wheel configuration.

In addition to the mature and immature forms, occasionally three more types of erythrocytes were noticed in the peripheral blood: microcytes, macrocytes and effete cells.

Microcytes are smaller than the mature erythrocytes and spherical in shape. Macrocytes are much bigger than the normal cells. They are usually round, or

occasionally slightly irregular cells with very pale pink cytoplasm and peripherally shifted, small, nearly round, light purple or pink, diffused nuclei usually with rugged margin.

Effete cells are large spherical or irregular cells with hyaline cytoplasm (which may be rarely very pale pink) and with centrally placed large nuclei usually presenting a characteristic trident arrangement of chromatin. The nucleus stains diffuse blue with the trident chromatin condensation presenting a bright purple color.

Anisocytosis of mature erythrocytes was also noted especially in the thicker regions of the smear. Similarly towards the periphery of the smear, the erythrocytes were swelled up; such cells were highly irregular in shape, had pale pink cytoplasm and swelled up, round cells with the pink chromatin arranged in the form of a very loose network; they differed distinctly from the effete erythrocytes in size, shape and staining reaction. Smudge cells and erythroplastids; erythrocytes without nucleus were also noted in the smears.

4.3.3 Thrombocytes

The second major category of cells found in the peripheral blood of *Etroplus maculates* are the thrombocytes. The average size of the cell is 6.17 X 2.93 μm and that of the nucleus, 4.12 X 2.4 μm. The size and the shape of this cell type, however show wide variation. The cells are slightly larger than the nucleus of erythrocytes. However they can be the same size as the erythrocyte nucleus.

Thrombocytes constitute 50 % to 54 % of the total leukocyte population (actual count: 31.7 X 10 3 to 35.3 X 10 3 mm3).

The cell shape is mainly provided by the nucleus which takes a variant of purple colour. However, in most blood film preparations the normal thrombocytes are few and irregular in shape due to their function in clotting; the thrombocytes may be grouped into three major categories based mainly on the shape of the cell and the nucleus shape: the spherical/oval forms, the oblong/elongated or spindle forms and tear drop shaped and irregular shaped forms. The first two categories abundant in the thrombocyte population in the peripheral blood; irregular forms

sparsely distributed. Tongues of cytoplasm may be observed at one or both ends of the cell. The thrombocyte has been referred to as spindle cells.

The cytoplasm of thrombocytes stains pale pink to pale blue color with MG-G stain. The pinkish hue is especially evident in the cytoplasm of cells with unipolar/ bipolar cytoplasmic extensions. The cytoplasm of oblong cells, in which it forms a complete rim around the nucleus, is usually hyaline. Blue colored cytoplasm has been noted in elongated and elongate irregular forms.

The nuclei of thrombocytes stain evenly deep purple with MG-G. Compared to the nuclei of lymphocytes, those of thrombocyte appear smooth and dense there being no appreciable clumping of chromatin.

Of the spherical/oval forms those with a perfect round shape are very few in number. In the majority of the spherical forms, the cytoplasm is indistinguishable. In some a clear cytoplasmic rim is discernible. The shape of, particularly the subspherical/ oval forms may be modified by the disposition of the cytoplasm. In some the cytoplasm is drawn out into a blunt cone or cap at one pole. In some others, a similar cytoplasmic extension may be present at the opposite pole also. The shape of the oval forms may also be modified by the presence of cytoplasmic extensions. Thus, comet shaped, tear-drop shaped, spindle shaped or cells with filamentary cytoplasmic extensions are noticeable in the peripheral blood.

The typical oblong/elongated thrombocyte has an elongate oval nucleus and a hyaline cytoplasmic rim of even width around the nucleus. In a few, a spike-shaped cytoplasmic extension at one pole is clearly visible.

Irregular forms are found both among the spherical /oval and oblong/elongated categories; the latter are more. Irregular spherical/oval forms are sometimes very difficult to distinguish from the small lymphocytes. However they are distinctly smaller than the small lymphocytes. Irregularity of elongated forms is because of the bizarre shape of the nuclei.

Thrombocytes generally show a tendency to clump together especially along the periphery of the blood film. This tendency of clumping is especially evident in

the case of spherical/oval forms; majority of the oblong/elongated and oblong-irregular cells are distributed solitarily.

The thrombocytic granules were dark or deeply basophilic in early thrombocytes lightly basophilic in the intermediate thrombocytes and reddish pink in the definite thrombocytes.

4.3.4 Leukocytes

4.3.4.1 Agranulocytes

Agranulocytes includes lymphocytes and monocytes.

Lymphocytes

Lymphocyte is the most common leukocyte type present in the peripheral blood of *Etroplus maculates*. It is usually round in shape with round nucleus. The cytoplasm forms a complete or partial rim around the nucleus. Because of the psudopodial extensions of the cytoplasm, the shape of the lymphocytes varies considerably from round-stellate to highly irregular. The average size is 5.73 X 3.4 μm with the nucleus measuring on an average 4.46 X 3.6μm. The size is variable. Among the lymphocytes large, medium and small lymphocytes are present. The lymphocytes are similar to their mammalian counterpart. The large lymphocytes have varied shapes from circular to an amoeboid form with pseudopod like formations on the cytoplasm. The cytoplasm takes a light basophilic stain. The nucleus is more lightly stained and takes a variant of magenta compared to the erythrocyte or thrombocyte nucleus.

Cytoplasm of lymphocytes stains pale to deep blue. In many cells cytoplasmic condensation, especially at the distal part of the peuodopodia, has been evident. The cytoplasm presents a coarse appearance. The nucleus stains purple or occasionally dark blue.

Large lymphocytes constitute 3 to 6 % (2.6 X 10 3-3.8 X 10 3 mm3) of the total leucocyte population. The average size of the cell is 11 μm and that of nucleus is 9.5 μm; the n-c ratio is 1: 1.15

Small lymphocytes. Their cell size is the same as that of thrombocytes (6.0um) but the n-c ratio is more in favour of the nucleus (nuclear diameter= 5.2 um cell diameter = 6.0 um n-c ratio is = 1 : 10. With Giemsa the nucleus shows variant of auricular purple stain which is similar to that of the thrombocytes. The main criteria by which the small lymphocytes can be distinguished from thrombocytes are: (1) the cytoplasm though less in volume is stained a light bluish with a basophilic dye; (ii) nucleus is more varied in shape (iii) small lymphocytes constitute only 2-5 % (actual count 1.4 X 103-2.8 X 10 mm3) compared to some 40 % thrombocytes; (iv) small lymphocytes are stable cells and do not change their morphology and pattern of distribution when the blood film is prepared. Small lymphocytes outnumber the other types of lymphocytes.

Majority of the medium sized lymphocytes are spherical (rarely oval). The cytoplasm present as an irregular rim all around or as a broad, irregular extension on one side or disposed in such a way as to impart a stellate appearance. They stain deeper than the large ones.

Monocytes

Monocytes were found to be the largest of the formed blood elements. Monocytes are distinguished from other granulocytes by their large average size (Cell diameter is 11.5 X 4.5 μm, nuclear diameter 6.2 X 4.54 μm, n-c ratio is 1: 1.80. The cytoplasm takes little stain even when exposed to Giemsa stain. The cytoplasm is deeply basophilc with MG-G stain. The nucleus has a variant of purple stain. The cells generally seen on the border of blood films surrounded by erythrocyte [plate. Monocytes are rare in the peripheral blood of the *Etroplus maculates*. They represent 5 % of the total leukocyte population in *Etroplus maculates*. (Counts 1.0 X 10 3 – 1.7 X 103 mm-3). The cell shape although variable is generally globular but the nucleus has variable forms.

The monocytes are large, oval or irregularly-shaped cells with eccentrically placed nuclei. The nucleus is usually hemispherical or in some cases, irregular shaped.

4.3.4.2 Granulocytes

Neutrophils

The cell which comes nearest to monocytes in its shape and size is the neutrophil, but it is comparatively smaller and has lesser n-c ratio. Neutrophils are more numerous compared to monocytes and their cytoplasm is clearly stained and granular. Neutrophils are generally subspherical to round cells with eccentrically placed nucleus. [plate a, b]. Their cytoplasm contains fine granules and almost transparent pale pink / pale blue appearance with MG-G stain. Nucleus has a variable form, from spherical to dumb bell shape. The nucleus shape is highly variable, elongated, horse shoe shaped, kidney/bean shaped. A few bi nucleate neutrophils and on occasion a cell with typical multilobed nucleus very similar to that found in human blood were also observed. Neutrophils are large cells, their mean diameter is 9.5 μm and that of nucleus is 5.8 μm, the n-c ratio is 1: 1.70. Compared to monocytes, neutrophils are only slightly smaller in size and the relative n-c ratio, but they are more numerous representing 20-32 % of the total leucocyte population. The total number of neutrophils vary from 12.3 X 103 to 18 .8 X 10 3 mm-3. The cytoplasm

Eosinophils

Eosinophils are typically amoeboid cells. The nucleus has an irregular form. Cytoplasm is acidophilic and stains pink and the acidophilic granules in the cytoplasm are evident. Eosinophils are minority being only 3-6 % of the total leucocyte population (total number varies from 2.2 X 10 3 – 2.9 X 10 3 mm3) The n-c ratio is I : 1.80, thus they have some morphological resemblance to monocytes, but the acidophilic nature of the cytoplasm helps to distinguish between the two cell types. Both in the nucleus and cytoplasm granules are seen. This feature is common to other granulocytes.

Basophils

Basophils are rounded and can be distinguished from other cell types by the bluish cytoplasm. The nucleus is generally rounded and often showed considerable variation. The chromatin of the nucleus is reticular and takes a variant of purple stain. A feature is the presence of one or more vacuoles in the nucleus and cytoplasm. Basophils represent a small minority of total leucocyte population being only 2-5 % (total number varies from 1.6 X 10 3- 2.9 X 10 3 mm-3). In size they equal to other granulocytes

The granular anucleated bodies were also observed in the blood smear.

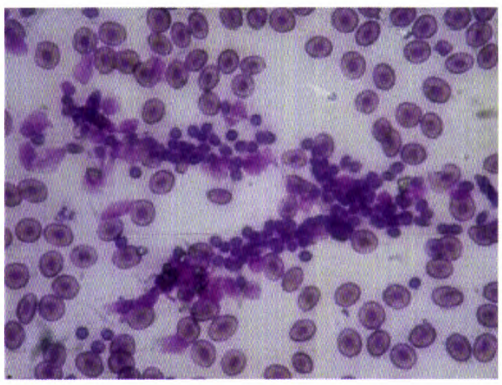


Fig. 4.1: Thrombocytes in Aggregation (20X)

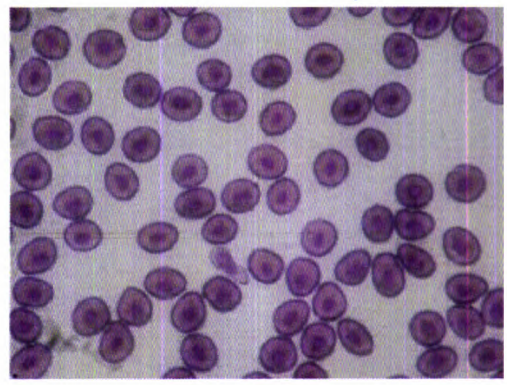


Fig. 4.2: Dumbell shaped Thrombocytes (20X)

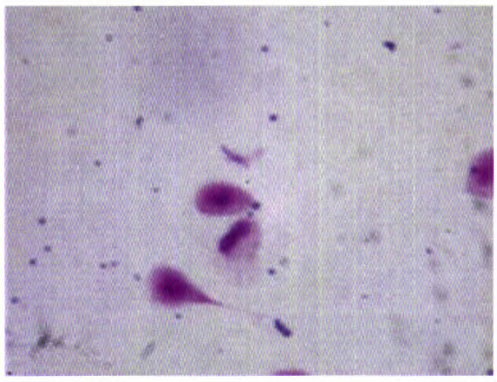


Fig. 4.3: Tear drop shaped Thrombocytes (20X)

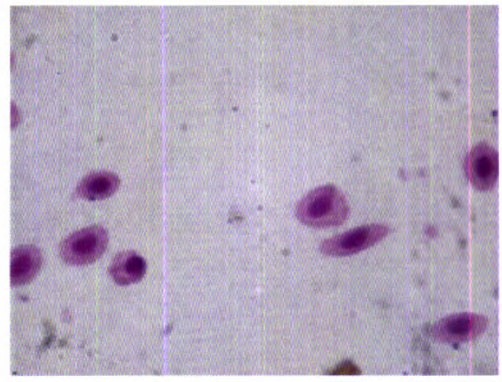


Fig. 4.4: circular Thrombocytes (20X)

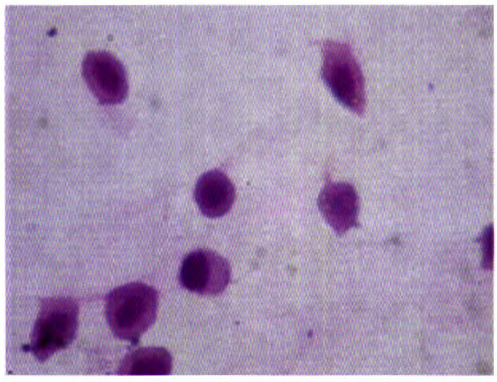


Fig. 4.5: Thrombocytes with Pseudopodia (40X)

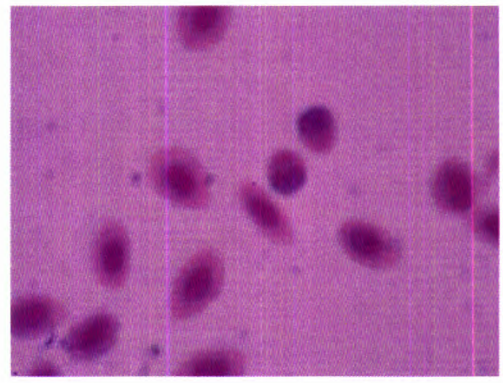


Fig. 4.6: Round Thrombocytes (40X)

Different types of Thrombocytes (Haematoxylin & eosin stain) (40X)

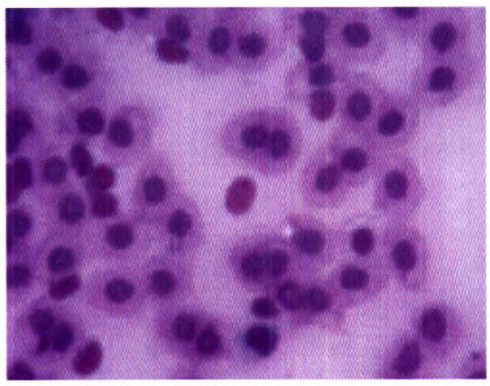


Fig. 4.7: Eosinophil (20X)

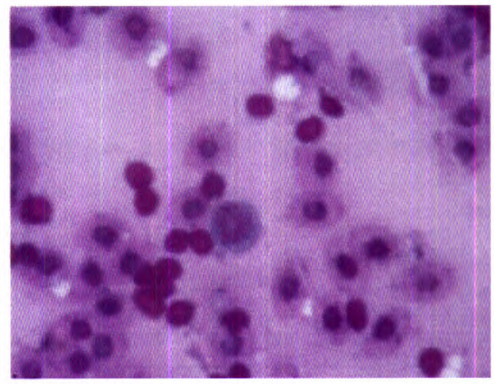


Fig. 4.8: Monocyte (20X)

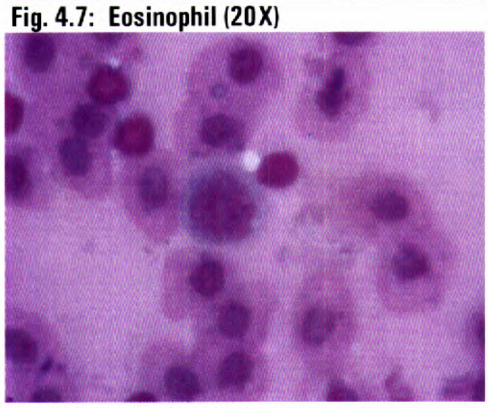


Fig. 4.9: Basophil (40X)

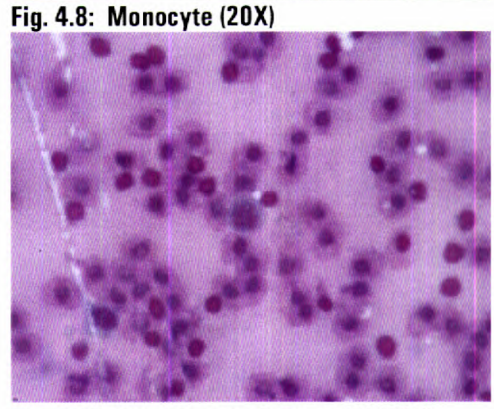


Fig. 4.10: Lymphocyte & Basophil (20X)

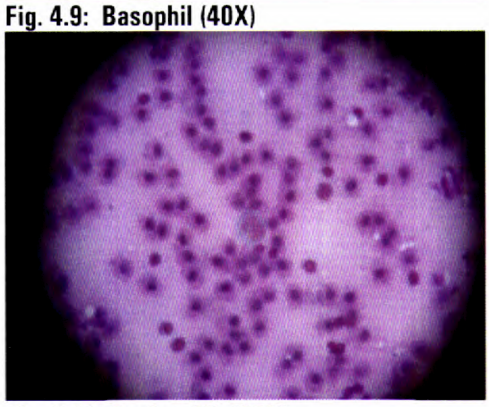


Fig. 4.11: Eosinophil (20X)

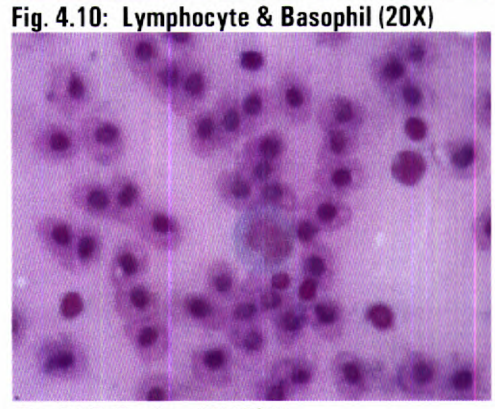


Fig. 4.12: Eosinophil (40X)

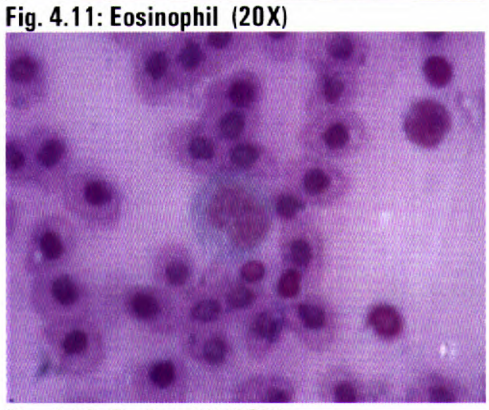


Fig. 4.13: Eosinophil (40X)

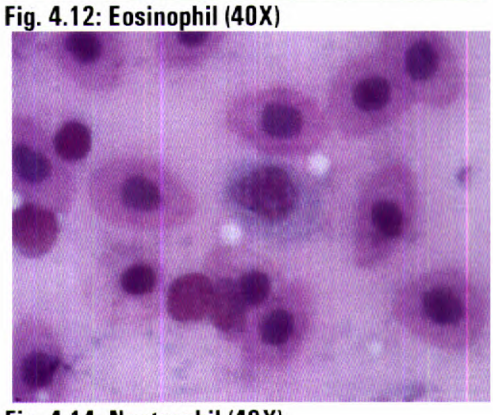


Fig. 4.14: Neutrophil (40X)

Different types of WBC (Haematoxylin & eosin stain) (40X)

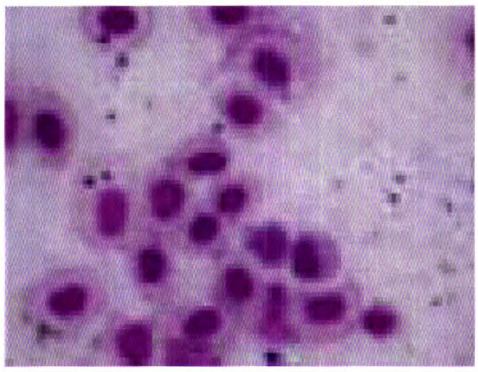


Fig. 4.15: Eosinophil (40X)

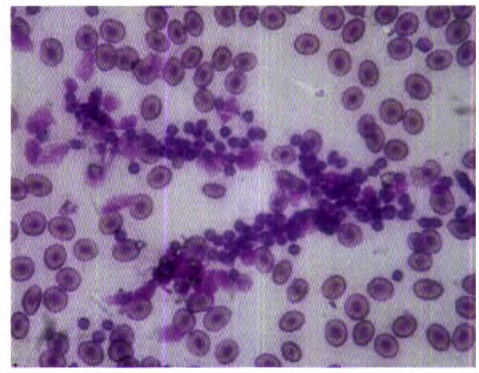


Fig. 4.16: Thrombocytes aggregation (20X)

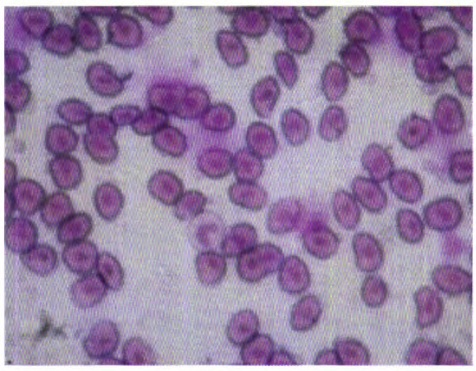


Fig. 4.17: Eunucleated RBC (20X)

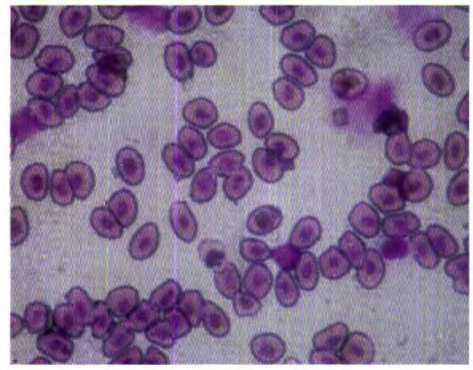


Fig. 4.18: Thrombocytes (20X)

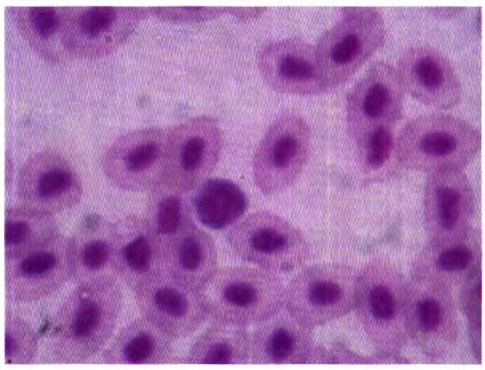


Fig. 4.19: Large Lymphocytes (40X)

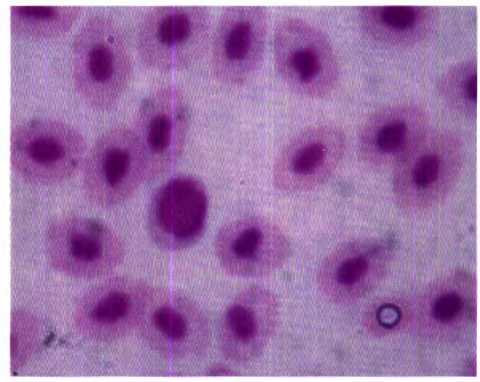


Fig. 4.20: Large Lymphocytes (40X)

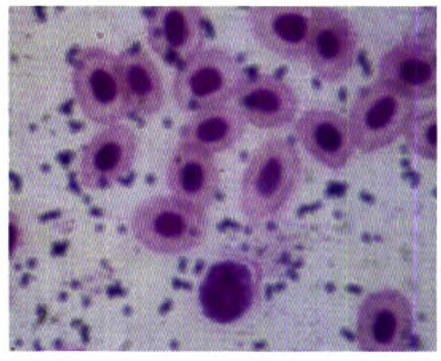


Fig. 4.21: Large Lymphocytes (40X)

Different types of WBC (Haematoxylin & eosin stain) (40X)

4.3.4.4 Enzyme cytochemistry

Results of the enzyme cytochemical tests are presented in Table

Table

Cytochemical staining reactions of *Etroplus maculates* blood cells

Enzyme	Erythrocyte	Lymphocyte	Monocyte	Neutrophil	Eosinophil	Basophil	Thrombocyte
PER	-	+ +	-	-	-	-	+ + +
ACP	-	+ + +	-	+ +	-	-	-
ALP	-	-	-	-	-	-	-
ANAE	-	+ + +	+	+ +	-	-	-/+
ASDE	-	+ +-	-	+	-	-	-

Enzyme positivity was indicated by the following

Peroxidase (Perox.) - Greenish –blue granules in cytoplasm

Alkaline phosphatase (Alk.phos.) - Red or deep brown –black precipitate uniformily throughout the cytoplasm.

Acid phosphatase (Ac.phos.) - Pink or reddish granules in cytoplasm.

α -naphthyl acetate - Dark red granules in cytoplasm

α-naphthyl acetate esterase (ANAE) - Dark red granules in cytoplasm

Naphthol-AS-D chloro acetate esterase - Bright red granules in cytoplasm (ASDE)

Cytochemical reactions of various blood cells were as follows.

Erythrocytes - Both mature and immature erythrocytes were negative to all tests.

Thrombocytes - Thrombocytes showed positive for peroxidase. No or weak positivity to ANAE. But they were negative to all other tests.

Lymphocytes - Lymphocytes were positive for peroxidase and negative for alkaline phosphatase. But they were strongly

positive for acid phosphatase which was completely inhibited by tartaric acid. ANAE was strong and ASDE was moderate.

Monocytes - Monocytes were negative for peroxidase, alkaline phosphatase, acid phosphatase and ASDE. They were positive for ANAE only.

Neutrophils - Neutrophils were negative for peroxidase and alkaline phosphatase. They had uniform acid positive granules; the activity was moderate. The reaction was totally inhibited by tartaric acid. Neutrophils were positive for both ANAE and ASDE. ASDE activity was week to moderate. ANAE activity was stronger than ASDE.

Eosinophils - Eosinophils were negative for peroxidase, acid phosphatase, alkaline phosphatase, ASDE and ANAE.

Basophils - Basophils were negative for all tests

4.4 DISCUSSION

Monocytes have not been described in fish blood by many workers.

Mechanisms of specific immunity in fish, similarly to lower vertebrates, are significantly less developed and play markedly less important role than in birds or mammals (Stosik *et al.*, 2001; Tavares-Dias *et al.*, 2007). In contrast, fish have non-specific resistance system, which plays the basic role in the organism defense against pathogenic and environmental factors (Stosik *et al* 2001; Passantino *et al.*, 2005).

In fish, any infection with any organism activates the cellular and humoral immune system. This is followed by changes in circulating antibodies and percentages and absolute number of the different WBC (Boon *et al.*, 1990). Quality and quantity of leukocyte cells which are haematologic parameters and generally used to determine of immune reactions and disease (Ekingen, 1988; Cagirgan, 1990).

The aim of stringent health control values is to provide a fish pathologist with a choice of sensitive method to enable early detection of physiological deviations from standard form, which may signal the on come of disease. The numbers and proportions of leukocytes, and thrombocytes (organic defence cells) in circulation provide an important representation of defence cell distribution throughout the body. In B *orbignyanus* there was intrespecific variation in total thrombocyte count, and the mean values were similar to those in C*yprinus carpio* (Tavares-Dias *et.al.*, 2004) however lesser than those in the characid *piractus mesopotmaicus* (Tavares-Dias and Mataqueiro, 2004).

Differences in the WBC counts may be attributed to many factors, both biotic (such as age, season, maturity, pathogens) and a biotic (including water temperature, PH, dissolved oxygen content, sex or maturity stage) and in particular to stress (Tavares-Dias and Moraes, 2004; Tavares-Dias and Moraes, 2007; Pavlidis *et.al.*, 2007). There for the difference demonstrated in this study may be related to species, nutritional differences, and sex or maturity stage.

According to Bayir (2005) WBC count was affected by water temperature and reproduction period. In *Etroplus maculates* under investigation the high WBC during pre spawning period and warm months were explained with increasing energy needs for reproduction, and decreasing oxygen amounts. However, studies have shown that total leukocyte, neutrophil and mococyte ratios were higher in females at reproduction periods than in males. (Murray, 1984; Orun and Erdemi, 2002).

The phagocytic activity of leucocytes plays an important role in the defence mechanism in animals. The phagocytic process in mammalian leucocytes has been extensively studied for all cell types from cyto-and immunochemical as well as fine structural, view points.

A survey of the available literature discloses there are many descriptions of the cellular elements of fish blood, however there are discrepancies. The discrepancies are evidences by the absence of standardization of terminology (nomenclature), as well as techniques utilized in fish haematology. The terminology

of the blood cellular elements in this study tend to follow that used by Catton (1951) and Leib *et.al.*, (1953).

It is very important to establish a classification of leucocytes for pathological examination of diseased fish and for experimental study of inflammation or immunopathology. The main constituents of leukocytes in the circulating blood of rockfish are neutrophils, monocytes, thrombocytes and lymphocytes (Suzuki *et.al.*, 1983). Piscine leukocytes, particularly granulocytes, have been controversial (Tavares-Dias et.al., 2003; Tavares-Dias and Barcellos, 2005; Tavares-Dias, 2006b; Tavares-Dias and Moraes, 2007). Several species have blood neutrophils and others have only heterophils, while in a few fishes, both neutrophils and heterophils are present (Tavares-Dias and Moraes, 2004; Tavares-Dias and Barcellos, 2005; Tavares –Dias, 2006; Tavares-Dias and Moraes, 2006 a, b). Doggett and Harris (1989) identified lymphocytes, thrombocytes, monocytes granulocytes such as, Type I granulocyte and Type II granulocyte in the peripheral blood of *Oreochromis mossambicus*.

In *B. orbignyanus* the neutrophil/heterophil number was found to be higher than that described for the characid *P.mesopotamicus* (Tavares-Dias and Mataqueiro, 2004) while lymphocyte numbers were smaller, and monocyte count very similar. However, when compared to *Cyprinus carpio* (Tvares-dias *et,al*, 2004), the number of these leukocytes was different. The methods used for counting were alike, but these are different species and from different environments, and other factors should be considered since the leukocytes are immune cells traveling between and through tissues to the blood stream (Tavares-Dias and Mataqieiro). Moreover, the response to different stimuli from the environment may vary among individuals of a same species as well as among species. The presence of leukocytes relates to important characteristics of health status in fish and in many cases they are also helpful in immune system evaluation. Therefore, variations in the proportions of these defence cells in the blood are usually expected.

Hine *et.al* (1998) hypothesized that neutrophils and heterophils represent cellular types of two different lines that evaluated, and they appear today as alternative types in lower vertebrates of the zoological scale, thus heterophils

developed eosinophilic properties while neutrophils basophilc properties. Nevertheless, neutrophils and heterophils were found in *B. orbignyanus*, and they exhibited both properties, eosinophilic and basophilic. In addition, blood lymphocytes, and monocytes were observed in *B. orbignyanus*. The occurrence of granulocytes in fish has been subject of some controversy, including Bryconinae. Therefore, granulocytes that have been described as special granulocytic cells in Brycon sp. (Ranzani-Paiva, 1996) and B. amazonicus (Tavares-Dias *et.al.*, 1999) are in fact heterophils. Such identification problems can be caused by inadequate Romanosky staining (e.g. May Gruenwalds –Giemsa), when used for staining blood smears of these species. These findings seem indicate that presence of both heterophil and neutrophil seem to be a characteristic of species from *Bryconinae* family.

In *Brycon orbignyanus* (Tavares-Dias, 2006a, b) as well as in *B.amazonicus*, no special granulocytic cell was found. Nevertheless, neutrophils extremely fine granules were found following staining with May Grunwald-Giemsa –Wright stain. Therefore, the misidentification of special granulocytic cell in *Brycon amazonicus* and *B.hilari* from literature. Can be due to failure in staining procedures of blood smears with May Gruenwald Giemsa stain, which makes a difficult cellular identification in some fish species. However, May Grunwald Giemsa-Wright stain allows easy recognition of heterophils and neutrophils. Other authors, for example, Tavares-Dias and Barcellos (2005) and Pavlidis *et.al* (2007), have also discussed misidentification founds resulting from the use of the May Gruenwald-Giemsa stain.

Barber *et.al.*, (1981) *Chaenocephalus aceratus* consists of small and large lymphoid cells, thrombocytes, both elongate / fusiform and round monocytes /macrophages, two types of granulocytes, one with rod like and other with spherical granule. Lymphoid cells show a range of sizes and morphologies which sometimes make their identification difficult. Small lymphocytes (6.81 X 6.55 um) and large lymphocytes (8.57 X 8.16 um). Lymphocyte nuclei occupy from 50 % to almost all the cell volume, with the larger cells having the greater proportion of cytoplasm. Nucleus usually eccentric, the degree of eccentricity depending on the

amount of cytoplasm. The cytoplasm is usually grayish blue to bright blue in MGG. The lymphocytes cells frequently exhibit pseudopodia. The smaller lymphocytes may be confused with rounded thrombocytes and the larger lymphocytes with monocytes / macrophages.

Fish leukocytes are involved in non-specific defense mechanisms (Tavares-Dias and Moraes, 2004; Tavares-Dias and Moraes, 2007; Pavlidis *et.al.*, 2007). Monocytes/ macrophages and lymphocytes are involved in the immune response leading to the production of antibodies. Neutrophils are effector key cells in non-specific immunity, as they migrate into the infection site, to recognize, ingest and destroy many pathogens (Tavares –Dias and Moraes, 2004; Tavares-Dias, 2006; Tavares-Dias and Moraes, 2007; Pavlidis *et.al.*, 2007). Heterophils and neutrophils have similar functions, but different morphological features (Tavares-Dias and Moraes, 2004).

Piscine thrombocytes represent a link between innate and adaptive immunity (Passantino *et.al.*, 2005). Therefore, piscine thrombocytes function is not limited to aggregation, but also may involve a great potential for phagocytosis (Kolman *et.al.*, 2003; Tavares Dias *et.al.*, 2007). In *Etroplus maculates* there was intraspecific variation in total thrombocyte counts, and the values were similar to those of *Brycon amazonicus* , and the values were similar to those in young *Brycon orbignyanus* (Tavares-Dias and Moraes, 2006a). Doggett *et.al.*, (1987) identified two forms of thrombocyte. The fusiform cell and oval shaped cell. Ferguson (1976) suggested, may be analogous to the surface connecting system of the mammalian platelet. Unlike those of other species eg. Plaice (Ferguson, 1976), dog fish (Morrow and Pulsford, 1980), rockfish and rainbow trout (Suzuki, 1984), thrombocytes of *Oreochromis mossambicus* were not found to phagocyte either colloidal carbon or bacteria. Thrombocytes are assumed to be involved in the clotting process.

According to Barber *et.al.*, (1981) the peripheral blood smears contain two morphologies of thrombocyte: round and elongate/fusiform, Both kinds are abundant. Round thrombocytes are often difficult to differentiate from small lymphocytes. Round thrombocytes by their small size (6.37 X 5.41 um) and light

grey, usually ragged cytoplasm. Occasionally these cells are seen in clumps. In some preparations there were pycnotic nuclei, which consider being thrombocyte nuclei. The elongate /fusiform thrombocyte is less frequent, but easier to identify. The nucleus is oval and densely staining and the pale grey cytoplasm tapers from one or both ends of the cell; cell borders are usually indefinable.

Thrombocytes are the un nucleated homologues of the human blood platelets (Watson *et.al* ., 1956) and their functions are in the blood clotting processes (Gardner and Yevich, 1959). Comparative morphological descriptions of the thrombocytes and lymphocytes have been provided by Saunders (1968a) The size characteristic has been reported by Catton (1951) and Saundes (1966). Descriptions of thrombocyte shape similar to as in *Etroplus maculates* given by Catton (1951), Sabins and Rangnekar (1962), Mc Knight (1966) and Saunders (1968a). The thrombocyte nucleus of Etroplus maculates stains more densely than the nucleus of a lymphocyte giving it a more compact appearance. This staining characteristic appear similar for the thrombocytes of other fish species (Gluckman and Gordan, 1953; Sabins and Rangnekar, 1962; Saunders, 1968a). The nuclei of thrombocytes are variable in shape. These variations are consistent with reports in the literature, as being round, oval, bilobed or u shaped (Catton , 1951); Boyar, 1962; Saunders, 1968a; Blaxhall and Daisley, 1973).

Lymphocytes were small round cells with moderately developed filopodia and abundant in the circulating blood. The nucleus was usually situated in the centre, but occasionally to one side of the cytoplasm and was round to oval, kidney shaped and bilobed. The nucleus was dark with an abundance heterochromatin area. Cells analogous to mammalian lymphocytes and macrophages have been observed in all major groups of fish (Good and Papermaster, 1964). In the channel cat fish, at least two types of cells classified as lymphocytes can be observed in whole blood preparations. One cell type referred to as a small lymphocyte is approximately the same size as and frequently confused with the thrombocyte. These cells can be distinguished from the thrombocyte which has a denser nucleus and less cytoplasm (Grizzle and Rogers, 1976: Saunders, 1968; Williams and Warner, 1976). The second lymphocyte cell type frequently referred to as the large

lymphocyte rangers in size from 7 to 11 microns. The eccentric nucleus, which is usually indented and very basophilic, occupies most of the cell. Small vacuoles and eosinophils granules are sometimes observed in the light blue cytoplasm, frequently, both types of lymphocytes are irregular and demonstrate pseudopodia.

The largest peripheral blood leucocytes were the monocytes, which generally displayed an irregular outline with occasionally a few pseudopodia. The nucleus occupied between a quarter and a third of the cell and although usually ovoid was sometimes reni form. Monocytes have been reported as absent from the blood of some species eg. Gold fish (Weinreb, 1963) and brown trout (Blaxha Daisley, 1973 and they are identified in a number of fish species (Ferguson, 1976; Parish *et,al.*). Monocytes fewer than neutrophils, were the largest cells in the circulating blood and their form was round to oval. Most nuclei were eccentric and showed plemorphism such as round, indented, kidney shaped or bilobed form.

Barber *et.al.*, (1981) opinioned that the monocytes /macrophages show a range of sizes and morphologies. Identifiable macrophages are the largest cells in the peripheral blood; monocytes are usually larger than cells consider large lymphocytes. The nuclei range from flattened and strongly eccentric to indent to horseshoe shaped. The cytoplasm of monocytes is typically grayish and flocculent and may contain un staining vacuoles. The macrophage is easily recognized when its usually basophilic cytoplasm contains staining heterophilic vacuoles. Macrophages usually have large PAS positive inclusions; monocytes are rarely PAS positive.

Most haematological research on fish blood indicates that monocytes were absent (Catton, 1951; Watson *et.al.*, 1956; Sabins and Rangnekar, 1962; Mc Knight, 1966; Saunders, 1968a; Blaxhall and Daisley, 1973). However, Gluckman and Gordon (1953) and Leib *et.al* (1953) report the presence of monocytes in the fish blood.. The monocytes in *Etroplus maculates* in most instances, as large as or larger than the erythrocyte. The cytoplasm stained a blue grey colour and was darker at the periphery of the cell and paled toward the nucleus. The cytoplasm stained a blue grey colour and was darker at the periphery of the cell and paled toward the nucleus. Vacuoles are present ranging from few and small to many and large. The

shape of the monocyte was consistently irregular and often characterised by the presence of psudopods..

The basophils examination of the literature reveals that this cell types was not found in a majority of the fish studied. Saunders (1966, 1968b) examined the formed blood elements of 171 species of fishes and observed five species in which this cell was present. In the *Etroplus maculates*, this cell was found infrequently. The basophil [plate] characteraized by the presence of small darkly staining granules. The nucleus of channel catfish basophil is eccentrically located and is somewhat oval in shape. The cytoplasm stains light blue, almost colourles (Williams and Warner, 1976).

The granular anucleated bodies were described by Catton (1951) in blood smears of the roach. *Rutiluus rutilus* and trout, but it has not been reported in other references. A few cell fitted the description of given by Catton were observed in some of the smears of *Etroplus maculates*.

Piscine leukocytes, particularly granulocytes, have been controversial (Tavares-Dias *et.al.*, 2003 ; Tavares-Dias and Barcellos, 2005; Tavares-Dias, 2006; Tavares-Dias and Moraes, 2007). Several species have blood neutrophils and others have only heterophils, while in a few fishes, both neutrophils and heterophils are present (Tavares-Dias and Moraes, 2004; Tavares-Dias and Barcellos, 2005; Tavares –Dias, 2006; Tavares-Dias and Moraes, 2006a, b).

The *Brycon amazonicus*, *B. orbignyanus* and *B. cephalus* had neutrophils and heterophils. neutrophils had typical granules while heterophils had eosinophilic–basophilc granules (Tavares-Dias *et.al.*, 2008). On the otherhand, studies in *Brycon amazonicus* (Carneiro *et.al.*, 2002) and *B. hilari* (Tavares-Dias *et.al.*, 2003) did not report the presence of heterophils, but only neutrophils.

Eosinophils and basophils were not recorded in the fish *Clarias gariepinus* (Gabriel *et.al.*, 2004). Reports of the presence of these cells in teleost fish are somewhat contradictory. Basophils were not found in the blood of plaice (Ellis, 1977), rainbow and brown trout (Blaxhall and Daisley, 1973). However, they were not recorded in some species like salmon pink (Ostroumova, 1960). Eosinophils are

usually rare in fish and their occurrence has been commonly recorded in haemopoietic tissues, for example in the kidney (Kelenyi and Neimeith, 1969).

In teleosts, the heterophil is the most frequent granulocyte. Neutrophils were round cells with an eccentric nucleus, and were common in the circulating blood. Most nuclei were band forms. But occasionally kidney shaped or bilobed. Fish heterophils have a variable staining of granules, which seem to depend on the species and / or cell maturity (Tavares-Dias and Barcellos, 2005; Tavares-Dias and Moraes, 2006 b)

Fish heterophil have a variable staining of granules, which seem to depend on the species and /or cell maturity (Tavares-Dias and Barcellos, 2005; Tavares-Dias and Moraes, 2006b; Tavares-Dias *et.al.*, 2008), and this fact has been also observed here in *Etroplus maculates*. In *Brycon amazonicus*, mature heterophils granules were identical to farmed *B. orbignyanus* described by Tavares-Dias and Moraes (2006b). However, it has been reported that in *B.orbignyanus* (Tavares-Dias and Moraes, 2006b), mature heterophil granules have few similarity to *Hoplosternum litoralle* (Tavares-Dias and Barcellos, 2005), but in both species the heterophils showed both eosinophilic and basophilic properties. Thus, it is easy to believe that the eosinophils reported for *B.hilari* (Tavares-Dias *et.al.*, 2003) and for *B.cephalus* (Arevalo and Castellano, 2003) may be immature .

The diagnostic characteristic of vertebrate basophils/ mast cells is the presence of metachromatic granules. However, they have been reported for only a few species of fish (Saunders, 1966; Ellis, 1977; in most species these cells have not been found, eg. (Drzewina, 1911; Saunders, 1966; Barber and Westermann, 1978a). In some species a PAS–positive granular leucocyte (PAS-GL), which has been interpreted as the teleost equalent of the basophil/mast cell, has been found (Barber and Westermann, 1975). Eosinophils, fewer than neutrophils, were round cells with an eccentric nucleus which was always round to oval and rarely lobed.

In the present study, *Etroplus maculates* granules were divided into three types based on the morphology findings. In a number of animals including fish species, neutrophils have been reported to possess several kinds of granules (Watanabe *et.al.*, 1967; Daems, 1968; Shively *et.al.*, 1969; Sonoda and Kobayashi,

1970; Akashi, 1976; Mainwaring and Rowley, 1985; Suzuki, 1986, Hine and Waain, 1988).

Barber *et.al.*, (1981) *Chaenocephalus aceratus* consists of small and large lymphoid cells, thrombocytes, both elongate /fusiform and round monocytes/macrophages, two types of granulocytes, one with rod like and other with spherical granule. Lymphoid cells show a range of sizes and morphologies which sometimes make their identification difficult. Small lymphocytes (6.81 X 6.55 um) and large lymphocytes (8.57 X 8.16 um). Lymphocyte nuclei occupy from 50 % to almost all the cell volume, with the larger cells having the greater proportion of cytoplasm. Nucleus usually eccentric, the degree of eccentricity depending on the amount of cytoplasm. The cytoplasm is usually grayish blue to bright blue in MGG. The lymphocytes cells frequently exhibit pseudopodia. The smaller lymphocytes may be confused with rounded thrombocytes and the larger lymphocytes with monocytes/macrophages.

Lymphocytes of *Horabagrus brachysoma* tested weakly positive for PER but negative for LAP, ANAE and ASDE (Prasad and Sonia, 2009). Karnowsky *et.al* (1981) suggested the efficiency of granulocyte PER in microbiocidal function. According to Hine *et.al* (1986a) and Meseguer *et.al* (1994), PER is not restricted to granulocytes. As peroxidases are the enzymes that destroy hydrogen peroxide, which is formed as a result of respiratory burst activity, the PER positive nature of lymphocytes suggests its role in the microbiocidal defence mechanism. Our observation of a lack of ALP in the lymphocytes of this species is consistent with the studies of Ellis (1976) and Ellariff (1982) and supported by the findings of Hine *et.al* (1986b) who suggested the absence of LAP in the lymphocytes of *Anguilla australis* and A. *dieffenbachia*. The lymphocytes of the channel cat fish, *Ictalurus punctatus* were also found to be LAP positive (Pitrie-Hanson and Ainsworth, 2000). Among different fish species, lymphocytes are reported to exhibit moderately positive reactions for ANAE and ASDE. Lymphocytes of *Cyprinus carpio* (Tripathi *et.al*, 2004), *Psetta maxima* (Burrows *et.al*., 2001) and the cat fish *Ictalurus punctatus* have been reported to be ANAE negative while those of *Scophthalmus maximus* (Burrows and Fletcher, 1987) and *Oreochromis*

mossambicus (Douggett *et.al*, 1987) were found to be ANAE positive. Among these species, only the lymphocytes of *Cyprinus carpio* were proved to be ASDE positive, although the other cell types were not studied for this enzyme.

Monocytes of *Horabagrus brachysoma* showed a positive reaction only to ANAE and were negative for the other three enzymes studied. The same enzyme pattern was found in the monocytes of *Astyanax bimaculatus*, *Hoplias malabaricus* (Tavares-Dias, 2006a), the armored catfish *Hoplosternum littorale* (Tavares –Dias and Barcellos, 2005) and the channel catfish *Ictalurus punctatus* (Petrie-Hanson and Ainsworth, 2000). Since nonspecific esterase plays an important role in the intracellular processing and trafficking of antigens, the nonspecific esterase-positive nature shown by monocytes of *H. brachysoma* maybe attributed to their participation in cellular defence, chiefly phagocytosis.

Neutrophils were found to be strongly positive for PER but devoid of activity for the other three enzymes tested in *Horabagrus brachysoma* (Prasad and Sonia, 2009). Peroxidase-positive reactions were observed in all forms of neutrophils, but predominantly in the bi-lobed cells. The presence of PER in neutrophils is an indication of their role in respiratory burst reactions associated with phagocytosis (Kemenede *et.al.*,, 1994). The same reaction occurs in all of the neutrophils of this species irrespective of the variations in cellular and nuclear morphology. The PER–positive nature of piscine neutrophils has been widely reported in *C. carpio* (Tripathi *et.al.*, 2004), *H. malabaricus*, *A. bimaculatus* (Tavares-Dias, 2006b), *P. maxima* (Burrows *et.al.*, 2001) and from catfishes such as *Ictalurus punctatus* and *H. littorale* (Tavares-Dias and Barcelos, 2005).

In the eosinophils and basophils of *Horabagrus brachysoma* a positive reaction could not be observed for any of the enzymes studied. A similar result was observed in the eosinophils of *H. littorale* (Tavares-Dias and Barcellos, 2005). The inability to demonstrate any enzyme in eosinophils and basophils may be due to the immature stages of these cells or due to its insignificant role in the defence mechanism.

The positive reaction of thrombocytes to PER and specific esterase may be attributed to their role in the defence mechanism, as reported by Rowley *et.al.*.

(1988). The phagocytic activity of fish thrombocytes has been reported earlier (Hill and Rowley, 1996; Slierendrecht *et.al.*, 1995; Burrows *et.al.*, 2001; Stosik *et.al.*, (2001; Kolman *et.al.*, 2003). There fore, the thrombocytes of *Horabagrus brachysoma* can act as blood phagoytes that form one of the protective barriers and may be considered true digesting cells in fish because of the presence of PER and specific esterase enzymes although more investigations are needed to prove this.

Hine *et.al* (1998) hypothesized that neutrophils and heterophils represent cellular types of two different lines that evaluated, and they appear today as alternative types in lower vertebrates of the zoological scale, thus heterophils developed eosinophilic properties while neutrophils basophilc properties. Nevertheless, neutrophils and heterophils were found in *B. orbignyanus*, and they exhibited both properties, eosinophilic and basophilic. In addition, blood lymphocytes, and monocytes were observed in *B. orbignyanus*. The occurrence of granulocytes in fish has been subject of some controversy, including Bryconinae. Therefore, granulocytes that have been described as special granulocytic cells in Brycon sp. (Ranzani-Paiva, 1996) and B. amazonicus (Tavares-Dias *et.al.*, 1999) are in fact heterophils. Such identification problems can be caused by inadequate Romanosky staining (e.g. May Gruenwalds –Giemsa), when used for staining blood smears of these species. These findings seem indicate that presence of both h heterophil and neutrophil seem be a characterastic of species from *Bryconinae* family.

In *B.orbignyanus* the neutrophil/heterophil number was found to be higher than that described for the characid *P. mesopotamicus* (Tavares –Dias and Mataqueiro, 2004), while lymphocyte numbers numbers were smaller, and monocyte count very similar. However when compared to *Cyprinus carpio* (Tavares-Dias *et.al.*, 2004), the number of these leukocytes was different. The methods used for counting were alike, but these are different species and from different environments, and other factors should be considered since the leukocytes are immune cells traveling between and through tissues to the blood stream (Tavares-Dias and Mataqueiro, 2004). Moreover, the response to different stimuli from the environment may vary among individuals of a same species, as well as

among species. The presence of leukocytes relates to important characteristics of health status in fish and in many cases they are also helpful in immune system evaluation. Therefore, variations in the proportions of these defense cells in the blood are usually expected.

The white blood cells play a major role in the defence mechanism of the fish and consist of granulocytes, monocytes, lymphocytes and thrombocytes. Granulocytes and monocytes function as phagocytes to salvage debris from injured tissue and lymphocytes produce antibodies (Ellis el.al., 1978; Wedemeyer and Mcleay, 1981).

Our results in Etroplus maculate show varying intracellular enzyme compositions of leucocytes. Lymphocytes of Heteropneustes branchysoma tested weakly positive for PER but negative for LAP, ANAE and ASDE (Prasad and Sonia, 2009). Karnowsky *et.al* (1981) suggested the efficiency of granulocyte PER in microbiocidal function. According to Hine *et.al* (1986a) and Meseguer *et.al* (1994), PER is not restricted to granulocytes. As peroxidases are the enzymes that destroy hydrogen peroxide, which is formed as a result of respiratory burst activity, the PER-positive nature of lymphocytes suggests its role in the microbiocidal defence mechanism. Our observation of a lack of LAP in the lymphocytes of this species is consistent with he studies of Ellis (1976) and Ellariff (1982) and supported by the findings of Hine *et.al* (1986b) who supported by the findings of Hine *et.al* (1986b) who suggested the absence of LAP in the lymphocytes of *Anguilla australis* and A. dieffenbachia. The lymphocytes of the channel catfish, *Ictalurus punctuatus* were also found to be LAP positive (Petrie-Hanson and Ainsworth, 2000). Among different fish species, lymphocytes are reported to exhibit moderately positive reactions for ANAE and ASDE. Lymphocytes of *Cyprinus carpio* (Tripathi *et.al.*,2004), *Psetta maxima* (Burrows *et.al* 2001) and the catfish *Ictalurus punctatus* have been reported to be ANAE negative while those of *Scophthalmus maximus* (Burrows and Fletcher, 1987) and *Oreochromis mossambicus* (Douggett *et.al.*, 1987) were found to be ANAE positive. Among these species, only the lymphocytes of *Cyprinus carpio* were proved to be ASDE positive, although the other cell types were not studied for this enzyme.

Monocytes of *Heteropneustes branchysoma* showed a positive reaction only to ANAE and were negative for the other three enzymes studied (Prasad and Sonia, 2009). The same enzyme pattern was found in the monocytes of *Astyanax bimaculatus*, *Hoplias malabaricus* (Tavares-Dias, 2006a), the armored catfish *Hoplosternum littorale* (Tavares-Dias and Barcellos, 2005) and the channel catfish *Ictalurus punctatus* (Pitrie-Hanson and Ainsworth, 2000). Since nonspecific esterase plays an important role in the intracellular processing and trafficking of antigens, the nonspecific esterase-positive nature shown by monocytes of *Etroplus maculatus* may be attributed to their participation in cellular defence, chiefly phagocytosis.

In *Etroplus maculates*, neutrophils were found to be strongly positive for PER but devoid of activity for the other three enzymes tested. Peroxidase –positive reactions were observed in all forms of neutrophils, but predominately in the bi-lobed cells. The same pattern of activity was reported by Prasad and Sonia (2009) in *Hetropneustes branchysoma.* The presence of PER in neutrophils is an indication of their role in respiratory burst reactions associated with phagocytosis (Kemenede *et.al.*, 1994). The same reaction occurs in all of the neutrophils of this species irrespective of the variations in cellular and nuclear morphology. The PER positive nature of piscine neutrophils has been widely reported in *Cyprinus carpio* (Tripathi *et.al.*, 2004), *H.malabaricus*, *A. bimaculatus* (Tavares-Dias, 2006b), *P. maxima* (Burrows *et.al* 2001) and from catfishes such as *I. punctatus* and *H.Littorale* (Tavares-Dias and Barcellos, 2005).

In the eosinophils and basophils of *Etroplus maculatus*, a positive reaction could not be observed for any of the enzymes studied. A similar result was observed in the eosinophils of *H. littorale* (Tavares-Dias and Barcellos, 2005) and in *H. brachysoma* (Prasad and Sonia, 2007). The inability to demonstrate any enzyme in eosinophils and basophils may be due to the immature stages of these cells or due to its significant role in defense mechanism.

The positive reaction of thrombocytes to PER and specific esterase may be attributed to their role in the defense mechanism, as reported by Rowley *et.al.*, (1988). The phagocytic activity of fish thrombocytes has been reported earlier (Hill

and Rowley, 1996; Slierendrechi *et.al.*, 1995; Burrows *et.al.*, 2001; Stosik *et.al.*, 2001; Kolman *et al.*, (2003). Therefore, the thrombocytes of *Etropplus maculatus* can act as a blood phagocytes that form one of the protective barriers and may be considered true digesting cells in fish because of the presence of PER and specific esterase enzymes, although more investigations are needed to prove this.

4.5 CONCLUSION

To conclude, as haematology assessment is gradually becoming routine practice for aquaculture field and it needs accurate information for identification and control of stress situations and or / diseases in order to ensure healthy fish , the evaluation of blood parameters may be the quickest way to detect these systems.. In summary the present study suggests that the presence of granulocytes, neutrophils and heterophils are characteristic to cichlids. Furthermore, leukocytes are the primary line of immunological defense and in healthy *Etroplus maculates*, lymphocytes and neutrophils were typically the most prevalent circulating leukocytes and there fore, these two granulocytes constitute the primary barrier of the immune system for this species. Hence, this immune function might be reflected by the lymphocytes and neutrophil counts, and then a lower number of these cells might result in a less efficient functioning of the immune system in *Etroplus maculates*. Thrombocytes are considered to have the additional function of haemato-plug formation during blood clotting. These properties reflect the effective immune mechanism, habit, habitat, evolutionary position and especially, the highly adaptive nature of this fish. Further studies are needed to improve our understanding of the functional role of fish thrombocytes in defense mechanisms.

Chapter 5

Heavy Metal induced changes in haematological parameters

HEAVY METAL INDUCED CHANGES IN HAEMATOLOGICAL PARAMETERS

5.1 INTRODUCTION

Aquaculture urges for more accurate information on stress control, in order to ensure health of fish under culture environment. The study of the physiological and haematological characteristics of cultured fish species is an important tool in the development of aquaculture system, particularly in regard to the use in detection of healthy from diseased or stressed animal (Rainza-Paiva *et al.*, 2000; O'neal and Werich, 2001).

Fish blood is a pathophysiological indicator of the whole body function and therefore blood parameters are important in diagnosing the structural and functional status of fish exposed to a toxicant (Sampath *et al.*, 1998).

The effect of heavy metals on aquatic organism especially fishes, is currently attracting wide spread attention particularly in studies related to industrial pollution. High toxicity of industrial pollutants have been known since long time, but their hazardous nature as pollution of aquatic environment has been matter of concern only after large number of deaths of fishes occurring indifferent areas due to different metals.

Fishes may be confronted with stress factors such as varied water qualities, pollution, malnutrition and disease. Fishes can adapt themselves to bad environmental conditions by changing their physiological activities. Blood tissue reflects physical and chemical changes occurring in an organism, there fore detailed information can be obtained on general metabolism and physiological status of fish in different groups of age and habitat (Kocabatmaz and Ekingen, 1978). Qualitative and quantitative variations in haematological parameters including the red blood cells (RBC) and white blood cells (WBC) numbers, cell proportions of leukocyte, the amount of hemoglobin (Hb), haematocrit (Hct) and the size of RBC and WBC

are the most significant findings as regards diagnosis. Consequently, the assessment of physiological parameters offer responses to toxicant stress (Luskova, 1998 and Omoregie, 1998)

Haematotoxicology is the study of adverse effects of drugs, nontherapeutic chemicals and other agents in our environment on blood and blood forming tissues Bloom, 1997). The vital functions that blood cells perform, together with the susceptibility of this highly proliferative tissue to intoxication, makes the hematopoietic system unique as a target organ. Hematotoxicity may be regarded as primary toxicity, where one or more blood components are directly affected. Primary toxicity is regarded as among the more common serious effects of xenobiotics (Vandendries and Drews, 2006).

Since haematological parameters reflect the poor condition of fish more quickly than other commonly measured parameters, and since they respond quickly to changes in environmental conditions, they have been widely used for the description of healthy fish, for monitoring stress responses and for predicting systematic relationships and the physiological adaptations of animals.

The present study has been aimed to assess the effect of different sublethal concentrations of heavy metals Cadmium and lead on the haematology of *Etroplus maculates* (Bloch). The various parameters studies included Hb concentration, RBC Count, WBC Count, PCV and RBC constants.

5.2 MATERIALS AND METHODS

Healthy specimens of *Etroplus maculates* (Orange chromide) were collected from the local water bodies of kothad, Kadamakudy area and were transported to the laboratory in plastic bags. In the laboratory, fishes were kept in large plastic bowls containing 60 L of clean tap water and acclimatized for 14 days to the laboratory conditions. During which time they were provided with artificial pellet feeds and boiled rice. The size of the fish varied from 4.5 -6.6 cm in standard length and weight 5 -8.5 gms. Fish of both sexes were used without discrimination. The fresh water used had a pH of 7.0±0.45, temperature of 26 ± 3 C, dissolved oxygen content of 7.8ppm and salinity of zero ppt.

5.3 BIOASSAY METHOD

The bioassay method adopted in the present study were same as that of Doudoroff *et.al.*, (1953). Experiments were carried out in semi static renewal system based on APHA (2005). The lethal toxicity studies provide information about the relative lethality of a toxicant. This test is designed to determine the highest concentration of a pollutant that is sufficient to affect usually 50 % of a limited number of organisms. Though the LC 50 appears to be crude method its importance is highlighted by many workers (Duke, 1974; Buikema (*et.al.*, 1982). The acute LC 50 value of heavy metals Cadmium and Lead was determined in the laboratory using the semi-static method (UNEP/FAO/IAEA, 1989) and was found to be 12ppm for cadmium and 20ppm based on the Probit analysis (Finney, 1957).

After determining LC 50 96 hr value 3 sublethal concentrations of Cadmium chloride and lead nitrate were taken and 10 fishes were introduced in each concentration. For each sublethal exposure 3 replicates were maintained. Eventhough the water was changed everyday in the control and the treatment group, the concentration of cadmium chloride remained the same within the experimental period. *Etroplus maculates* was exposed to sublethal concentration of 0.33ppm, 0.67ppm and 1.2ppm. Cadmium chloride and 2 ppm, 4 ppm and 6.6 ppm of lead nitrate for 21 days. At the end of 7 th, and 21 st day sampling was done. Blood from the control and cadmium chloride and lead nitrate treated fishes were obtained by severance of caudal peduncle and collected in Eppendorf tubes containing 1 % of Ethylene diamine tetra acetic acid (EDTA) as anticoagulant (Mgbenka *et.al.*,.) 2003. Haematological parameters were estimated by standard methods. Detailed methodology as described in Chapter 1.

5.4 STATISTICAL ANALYSIS

Data were statistically analyzed by three factor ANOVA followed by LSD analysis, using statistical software SPSS-16.

5.5 MACROSCOPIC (BEHAVIORAL) OBSERVATION

Lead nitrate exposed *Etroplus maculates* display lower stamina. Pb decreases heme synthesis (Bolognani Fantin *et.al.*, 1989; Tabche *et.al.*, 1990) and erythrocyte concentrations (Dawson, 1930, 1933, 1935) in fishes with in a few days.

On exposure to sub lethal concentration of cadmium chloride, the toxic stress on the fish was manifested in the form of restlessness and jerky and erratic swimming movements. The exposed fish also showed increased entilator movements of operculam and increased gulping activity. Instantaneous secretion of excessive mucus all over the body surface of the exposed fish was also noticed. Copious amounts of mucus were later released into the media at various stages of exposure in the form of streaks, along with rejected flakes of epithelial cells and other cell debris. Even though the exposed fish rejected the food provided, especially in the earlier stages of exposure (up to 5 th day) they started consuming the food. With hesitation and gradually resumed feeding activity to near normal situation by the 12 th day. No death occurred in the experimental and control groups.

The results of the present investigation showed various anomalies in the blood of Etroplus maculates during prolonged exposure to Cadmium chloride and Lead nitrate. No significant changes were observed in the measured variables of fish maintained in uncontaminated water (control). The main haematological alteration resulting from exposure of *Etroplus maculates* sp. To various concentrations of cadmium I water for 21 days includes significant decrease in haematocrit and haemoglobin concentration and non significant decrease in red blood cell counts (Table 2). The white blood cell counts also decreased with a change in the composition as seen from the differential white blood cell counts (table 3)

In the present study, it has been observed that, in the exposed fished the number of RBCs and hemoglobin percentage decreased significantly from normal values. However differential leucocyte counts were deviating significantly from normal values. The increase was observed in the number of lymphocytes and eosinophils while decrease was noticed in the number of monocytes and neutrhils. This is in agreement with Mastan *et al.*, 2009, Shan, 2009 and Adeyemo *etal.*, 2009).

Descriptive Statistics for Cadmium Toxicity in Etroplus Maculatus

Concentration	Day	Experiment						
		Hb	PCV	RBC	MCV	MCH	MCHC	WBC
Control		8.30± 0.3105	23.36± 2.0787	3.07± 0.2276	78.08± 7.2385	27.27± 1.5309	35.53± 3.4873	3.58± 0.0684
0.33 ppm	7th Day	7.81± 0.3875	25.01± 1.9498	2.37± 0.3895	102.8± 20.8694	31.19± 4.5201	31.33± 3.1289	3.47± 0.0778
	21st Day	7.51± 0.4160	26.21± 1.8456	1.89± 0.6260	123.2± 34.8143	32.88± 8.8347	28.46± 3.0292	3.32± 0.0458
0.67 ppm	7th Day	7.12± 0.2973	27.63± 1.6335	1.43± 0.4170	159.8± 40.5251	41.22± 11.0451	25.68± 2.7404	3.20± 0.0599
	21st Day	6.55± 0.3136	28.93± 1.3865	1.14± 0.5404	194.3± 87.6247	44.53± 20.6385	23.03± 1.9419	3.08± 0.0919
1.2 ppm	7th Day	6.29± 0.2963	30.56± 1.2408	0.83± 0.6998	193.8± 19.4593	40.55± 6.8590	20.94± 1.4905	2.91± 0.0979
	21st Day	5.25± 0.2637	32.41± 1.0754	0.68± 0.6870	257.8± 144.613	35.28± 2.4512	16.07± 1.4003	2.47± 0.1034

Analysis of Variance for Cadmium Toxicity

Source	Sum of Squares	df	Mean Square	F	P-value
Experiment	1674.116	6	279.019	965.152	0.000
Error	218.555	756	0.289		
Total	1892.671	762			

Here the p-value is less than the significance value 0.05, we conclude that the experiment is statistically significant. That means the values of cadmium toxicity is varying with different experiments.

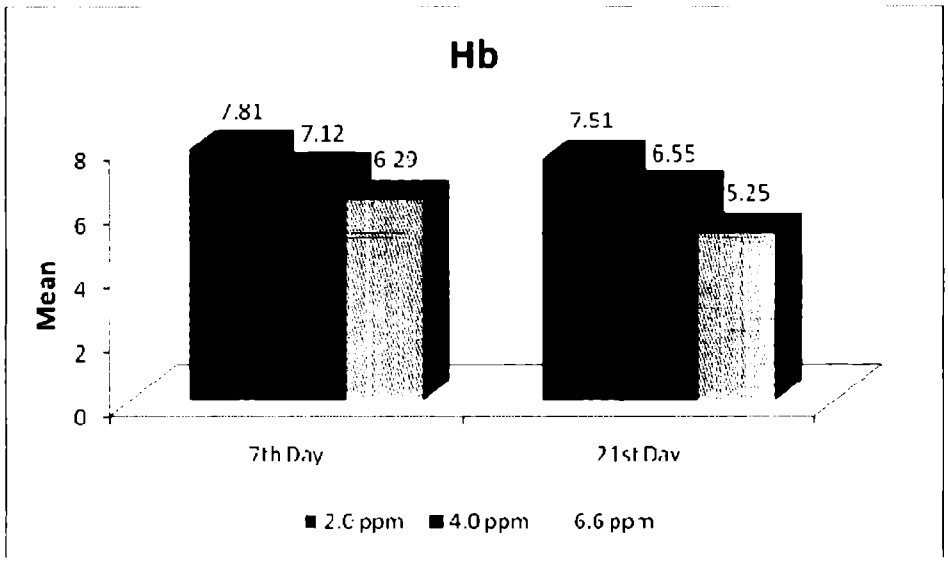
Pair wise Comparisons

	Mean Difference	p-value
Hb vs. PCV	-1.384	0.000 [S]
Hb vs. RBC	1.848	0.000 [S]
Hb vs. MCV	-3.033	0.000 [S]
Hb vs. MCH	-1.624	0.000 [S]
Hb vs. MCHC	-1.288	0.000 [S]
Hb vs. WBC	0.792	0.000 [S]
PCV vs. RBC	3.232	0.000 [S]
PCV vs. MCV	-1.649	0.000 [S]
PCV vs. MCH	-0.240	0.081 [NS]
PCV vs. MCHC	0.096	0.795 [NS]
PCV vs. WBC	2.176	0.000 [S]
RBC vs. MCV	-4.881	0.000 [S]
RBC vs. MCH	-3.472	0.000 [S]
RBC vs. MCHC	-3.136	0.000 [S]
RBC vs. WBC	-1.056	0.000 [S]
MCV vs. MCH	1.409	0.000 [S]
MCV vs. MCHC	1.745	0.000 [S]
MCV vs. WBC	3.825	0.000 [S]
MCH vs. MCHC	0.336	0.002 [S]
MCH vs. WBC	2.416	0.000 [S]
MCHC vs. WBC	2.080	0.000 [S]

Analysis of Variance for Hb

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	64.747	2	32.373	252.631	0.000
Day	11.046	1	11.046	86.202	0.000
Error	15.506	121	0.128		
Total	128.189	125			

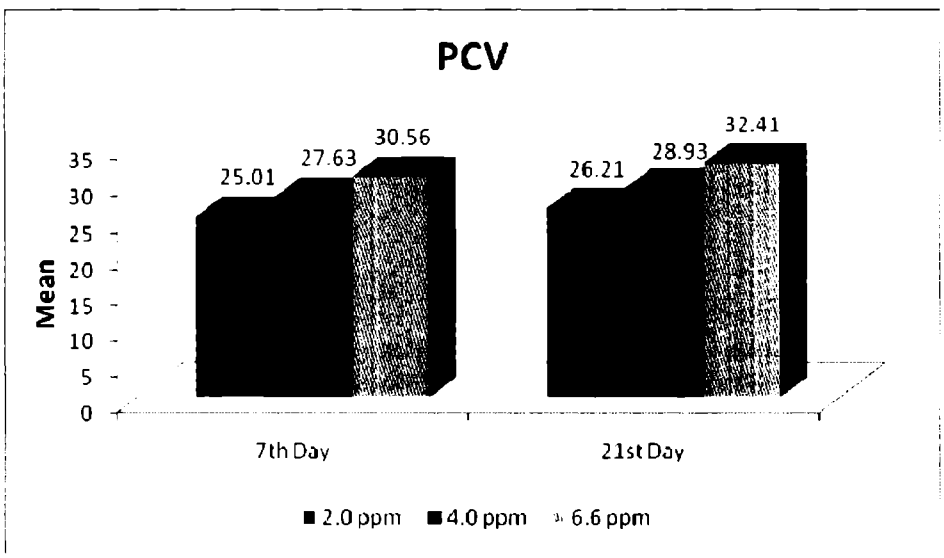
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of Hb is varying with different levels of concentration and different days.



Analysis of Variance for PCV

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	622.319	2	311.159	116.886	0.000
Day	56.666	1	56.666	21.286	0.000
Error	322.110	121	2.662		
Total	1402.028	125			

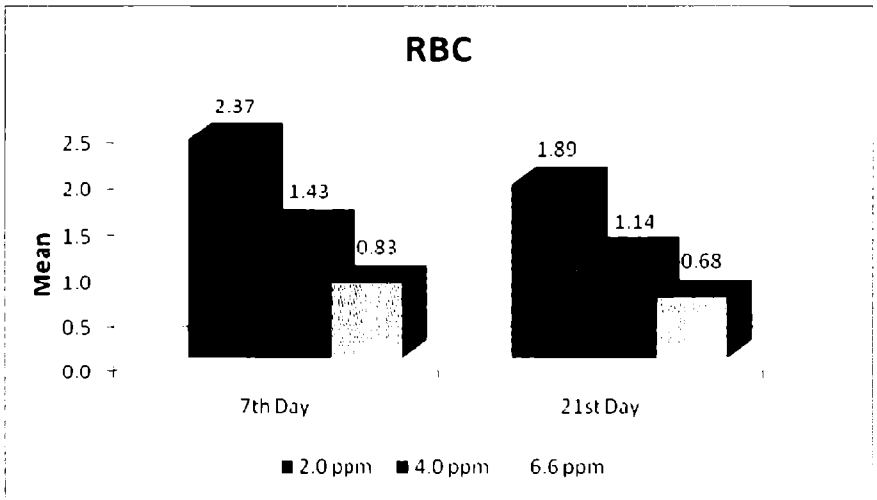
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of PCV is varying with different levels of concentration and different days.



Analysis of Variance for RBC

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	34.869	2	17.434	60.485	0.000
Day	2.575	1	2.575	8.933	0.003
Error	34.877	121	0.288		
Total	115.982	125			

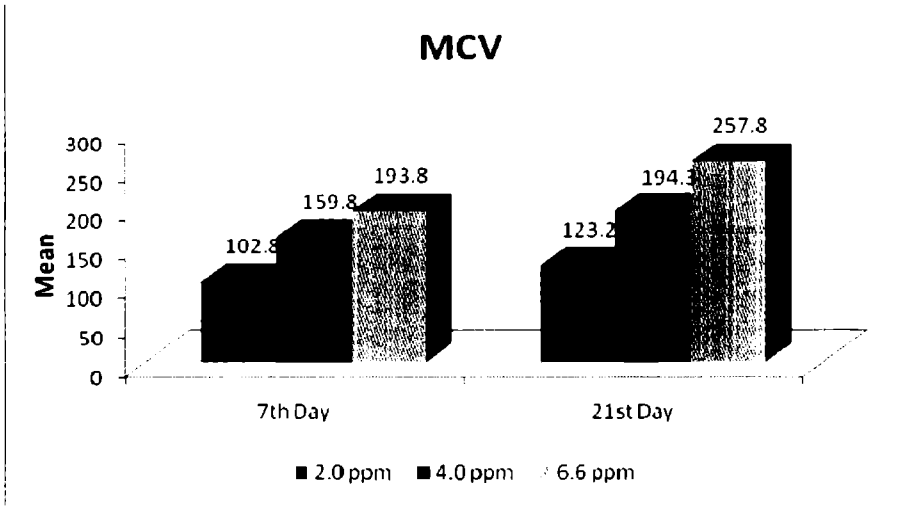
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of RBC is varying with different levels of concentration and different days.



Analysis of Variance for MCV

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	5.016	2	2.508	45.765	0.000
Day	0.532	1	0.532	9.700	0.003
Error	3.946	72	0.055		
Total	14.274	76			

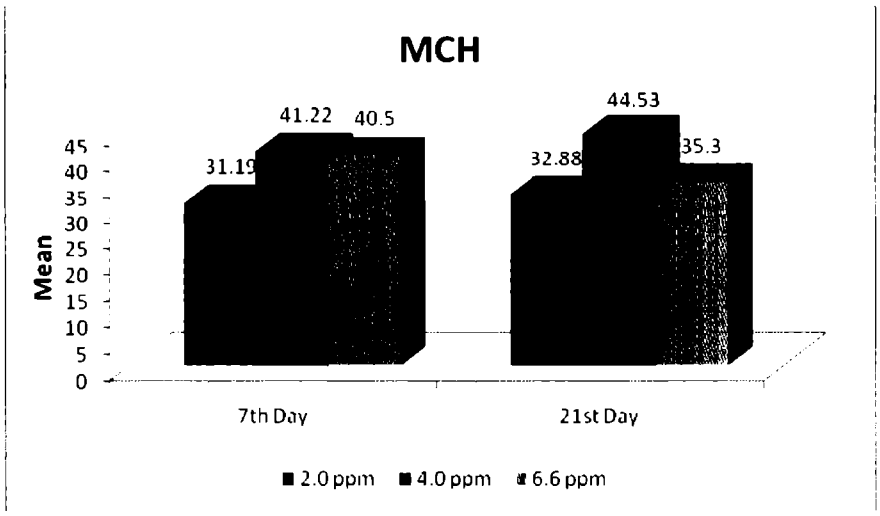
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of MCV is varying with different levels of concentration and different days.



Analysis of Variance for MCH

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	0.580	2	0.290	7.879	0.001
Day	0.004	1	0.004	0.105	0.747
Error	1.877	51	0.037		
Total	3.048	55			

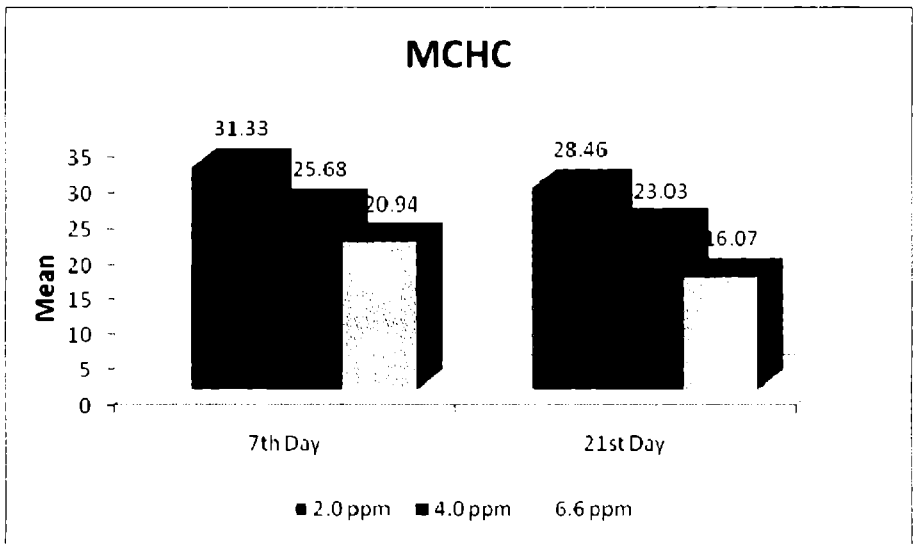
Here the p-value corresponding to concentration is less than the significance value 0.05, we conclude that concentration is statistically significant. However, it is noted that the p-value corresponding to day is greater than the significance value 0.05, we conclude that day is not statistically significant.



Analysis of Variance for MCHC

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	2337.153	2	1168.576	172.670	0.000
Day	323.822	1	323.822	47.848	0.000
Error	818.891	121	6.768		
Total	5443.649	125			

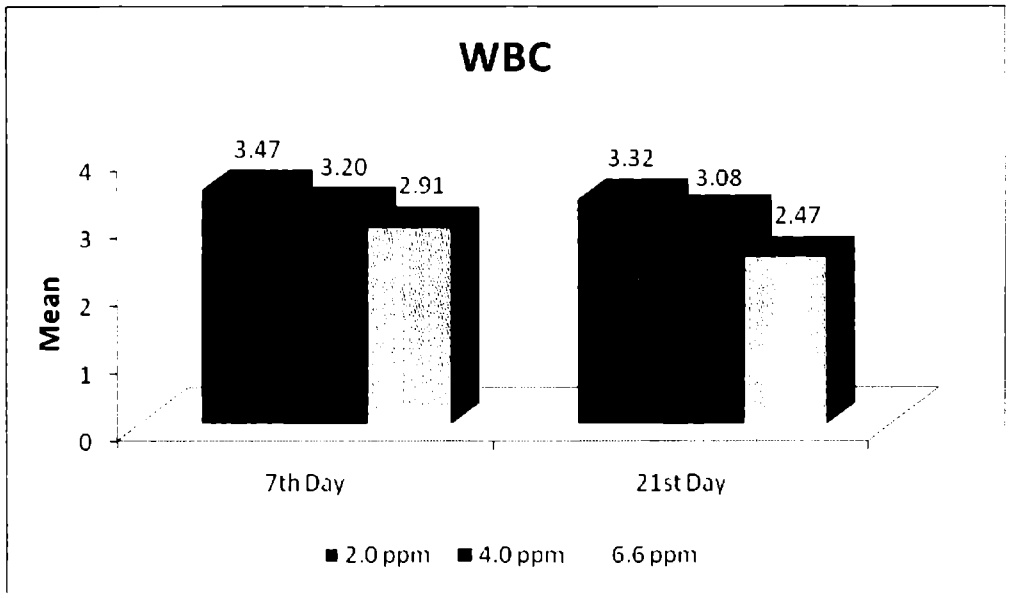
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of MCHC is varying with different levels of concentration and different days.



Analysis of Variance for WBC

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	9.055	2	4.527	410.644	0.000
Day	1.532	1	1.532	138.913	0.000
Error	1.334	121	0.011		
Total	15.822	125			

Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of MCHC is varying with different levels of concentration and different days.



Descriptive Statistics for Lead Toxicity

Concentration	Day	Experiment						
		Hb	PCV	RBC	MCV	MCH	MCHC	WBC
Control		8.27± 0.3292	23.42± 2.1511	2.97± 0.1072	78.91± 8.2087	27.87± 1.5989	35.59± 3.4566	3.58± 0.0684
2.0 ppm	7th Day	7.87± 0.3723	24.76± 2.3324	2.24± 0.4678	115.6± 28.7001	36.94± 9.4399	32.03± 3.2236	3.70± 0.0806
	21st Day	7.57± 0.4184	25.84± 2.3449	1.85± 0.6008	155.4± 51.3268	45.52± 14.9007	29.52± 3.0506	3.78± 0.0876
4.0 ppm	7th Day	7.19± 0.2905	27.15± 2.3095	1.42± 0.4560	211.8± 69.1579	55.99± 17.4861	26.69± 2.7201	3.68± 0.1658
	21st Day	6.70± 0.3219	28.41± 2.2792	1.15± 0.5460	306.4± 137.168	72.54± 32.6891	23.70± 1.9395	3.61± 0.1346
6.6 ppm	7th Day	6.43± 0.3020	30.00± 2.2440	0.85± 0.7660	1429.9± 1287.47	310.0± 279.981	21.51± 1.6581	3.18± 0.1271
	21st Day	5.39± 0.3186	31.66± 2.1460	0.74± 0.7385	2860.0± 2724.74	489.2± 467.988	17.09± 1.2554	2.84± 0.1907

Analysis of Variance for Lead Toxicity in Etroplus Maculatus

Source	Sum of Squares	df	Mean Square	F	P-value
Experiment	2537.027	6	422.838	719.002	0.000
Error	514.579	875	0.588		
Total	3051.606	881			

Here the p-value is less than the significance value 0.05, we conclude that the experiment is statistically significant. That means the values of lead toxicity is varying with different experiments.

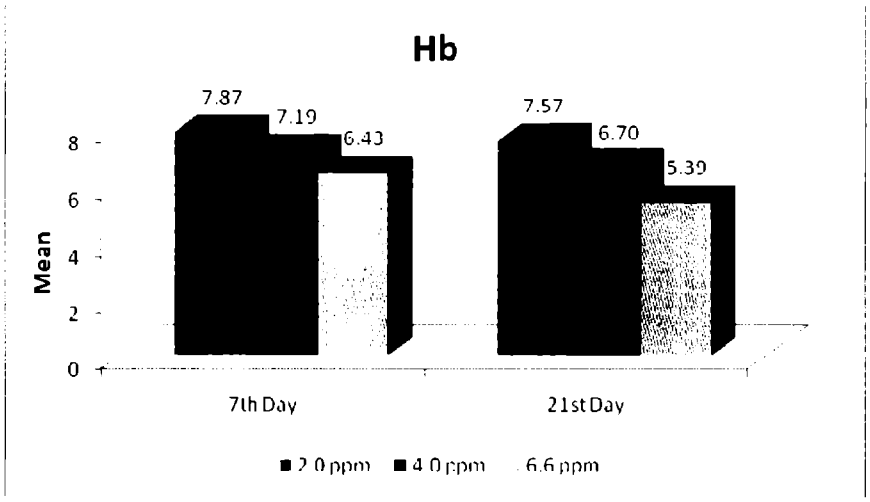
Pair wise Comparisons

	Mean Difference	p-value
Hb vs. PCV	-1.355	0.000 S
Hb vs. RBC	1.854	0.000 S
Hb vs. MCV	-3.566	0.000 S
Hb vs. MCH	-2.211	0.000 S
Hb vs. MCHC	-1.306	0.000 S
Hb vs. WBC	0.702	0.000 S
PCV vs. RBC	3.208	0.000 S
PCV vs. MCV	-2.212	0.000 S
PCV vs. MCH	-0.857	0.000 S
PCV vs. MCHC	0.049	0.999 NS
PCV vs. WBC	2.057	0.000 S
RBC vs. MCV	-5.420	0.000 S
RBC vs. MCH	-4.065	0.000 S
RBC vs. MCHC	-3.159	0.000 S
RBC vs. WBC	-1.151	0.000 S
MCV vs. MCH	1.355	0.000 S
MCV vs. MCHC	2.261	0.000 S
MCV vs. WBC	4.269	0.000 S
MCH vs. MCHC	0.906	0.000 S
MCH vs. WBC	2.914	0.000 S
MCHC vs. WBC	2.0082	0.000 S

Analysis of Variance for Hb

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	59.388	2	29.694	221.089	0.000
Day	9.986	1	9.986	74.350	0.000
Error	16.251	121	0.134		
Total	116.403	125			

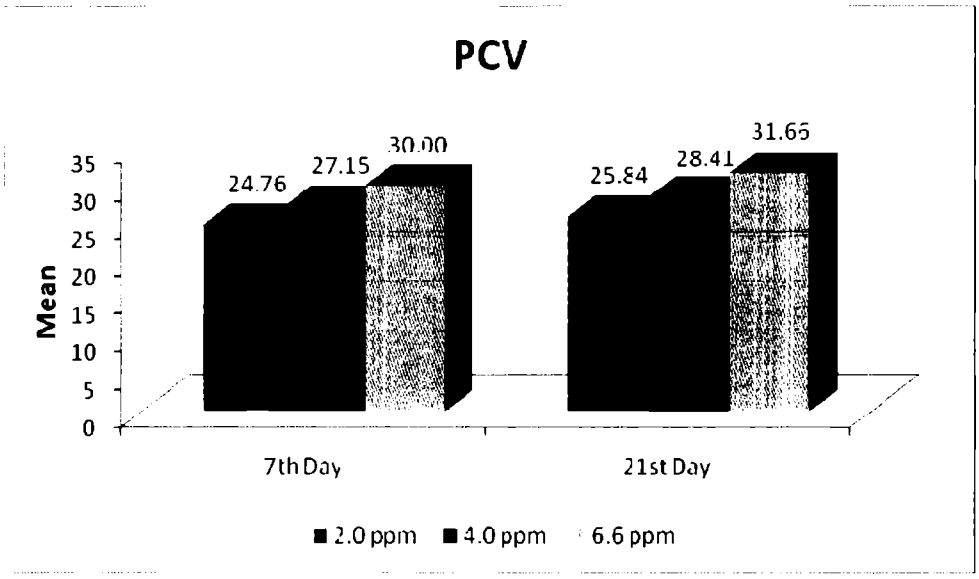
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of Hb is varying with different levels of concentration and different days.



Analysis of Variance for PCV

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	553.562	2	276.781	54.984	0.000
Day	47.987	1	47.987	9.533	0.003
Error	609.090	121	5.034		
Total	1530.712	125			

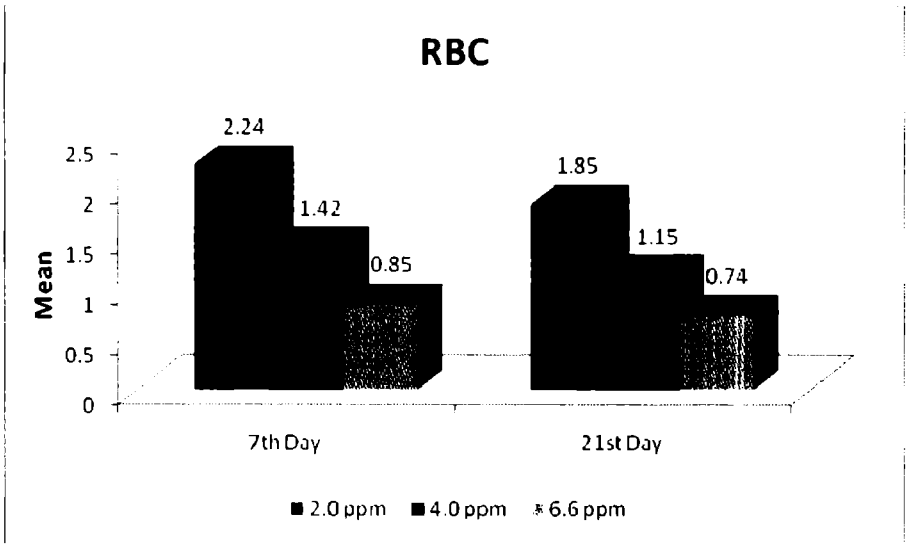
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of PCV is varying with different levels of concentration and different days.



Analysis of Variance for RBC

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	28.587	2	14.294	45.206	0.000
Day	1.794	1	1.794	5.675	0.019
Error	38.258	121	0.316		
Total	108.035	125			

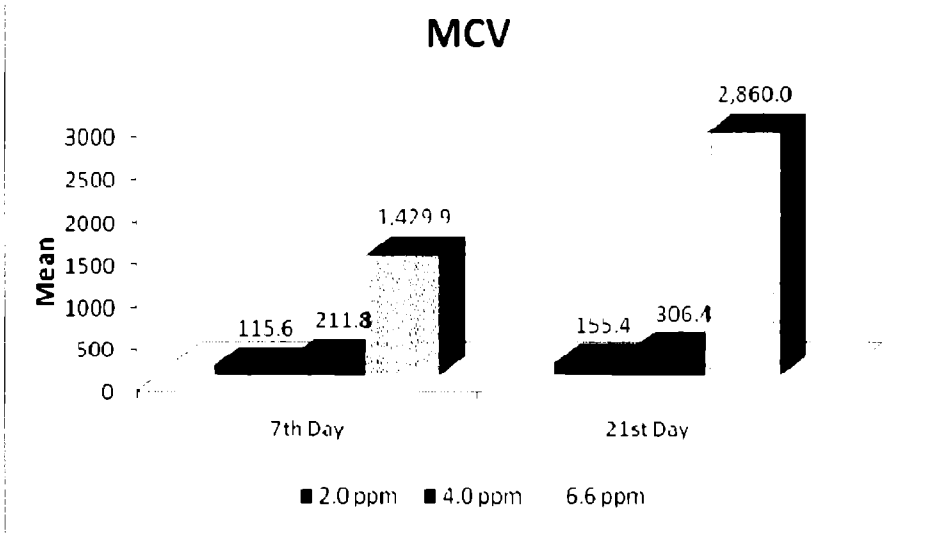
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of RBC is varying with different levels of concentration and different days.



Analysis of Variance for MCV

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	70.861	2	35.430	48.933	0.000
Day	3.303	1	3.303	4.562	0.035
Error	87.612	121	0.724		
Total	189.437	125			

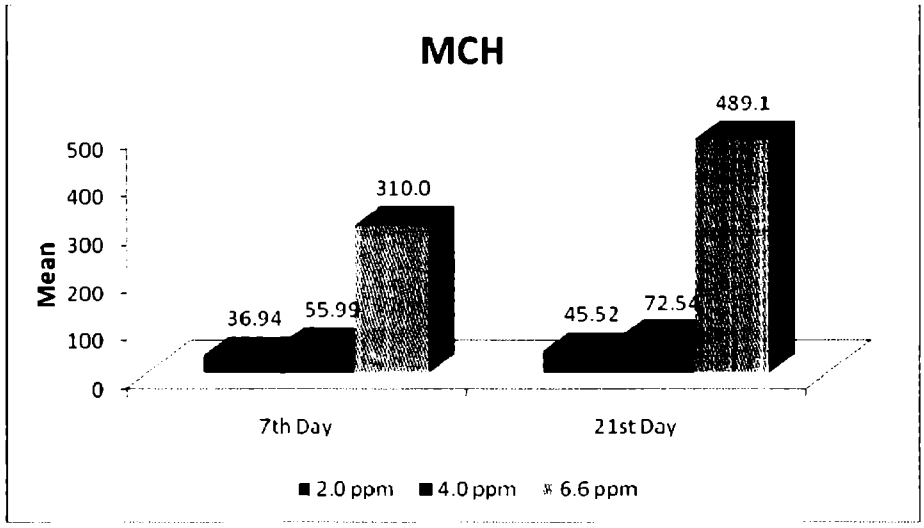
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of MCV is varying with different levels of concentration and different days.



Analysis of Variance for MCH

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	41.477	2	20.738	28.578	0.000
Day	1.148	1	1.148	1.582	0.211
Error	87.807	121	0.726		
Total	144.905	125			

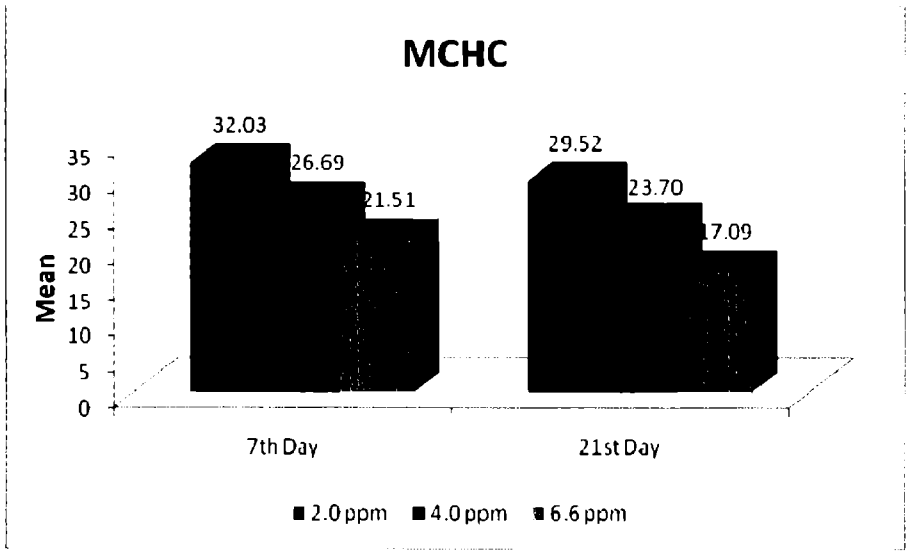
Here the p-value corresponding to concentration is less than the significance value 0.05, we conclude that concentration is statistically significant. However, it is noted that the p-value corresponding to day is greater than the significance value 0.05, we conclude that day is not statistically significant.



Analysis of Variance for MCHC

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	2370.647	2	1185.324	175.086	0.000
Day	295.252	1	295.252	43.612	0.000
Error	819.166	121	6.770		
Total	5185.855	125			

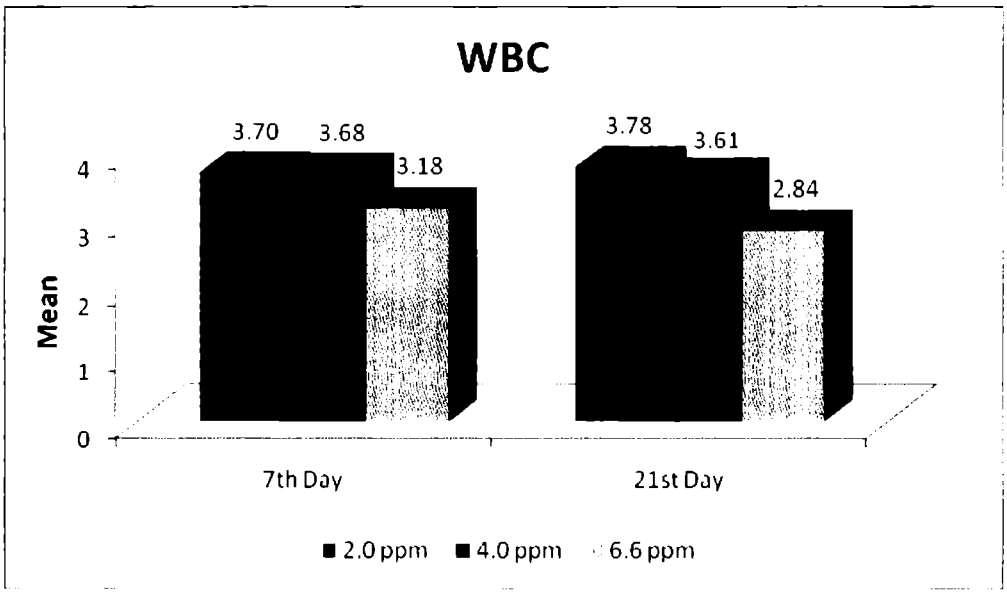
Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of MCHC is varying with different levels of concentration and different days.



Analysis of Variance for WBC

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	11.338	2	5.669	246.539	0.000
Day	0.320	1	0.320	13.902	0.000
Error	2.782	121	0.023		
Total	14.634	125			

Here the p-values corresponding to concentration and day are less than the significance value 0.05, we conclude that the concentration and day are statistically significant. That means the values of MCHC is varying with different levels of concentration and different days.



5.6 DISCUSSION

Aquaculture urges far more accurate information on stress control, in order to ensure health of fish under culture environment. Haematological parameters can be of great importance to fish farmers, serving as indicators of the physiological status and helping to prevent and control pathologies related to stress (Aldrin *et.al.*, 1982)

Contamination of aquatic environment by heavy metals whether as a consequence of acute and chronic events constitutes additional source of stress for aquatic organisms. Sublethal concentrations of toxicants in the aquatic environment will not necessarily result in outright mortality of aquatic organisms. Omoregie *.et.al.* (1990) reported that toxicants and pollutants have significant effects, which can result in several physiological dysfunctions in fish. Dysfunction in the fish induces changes in blood parameters possible as a result of blood water content.

In recent years haematological variables have been used more to determine the sublethal concentrations of pollutants (Wedemeyer and Yasutake, 1977). The use of immune system parameters to assess alterations in fishes experiencing heavy metal exposure and interest in defense mechanisms stem from the need to develop healthy management tools to support a rapidly growing aquaculture industry (Jones, 2001). The results of the present investigation showed various anomalies in the blood of *Etroplus maculatus*, during prolonged exposure to heavy metals Cadmium and lead.

The present study reveals that fish exposed to Cd alone showed significant reduction in RBcs, Hb and Hct than those of control. The reduction of these parameters at sub lethal level of cadmium might be due to the destruction of mature RBCs and inhibition of erythrocyte production due to the reduction of haemo synthesis that affected by pollutants. (Wintrobe, 1978). Gill and Epple (1993) found a significant reduction in the RBCs, Hb and Hct in American eel (*Anguilla rostrata*) after exposure to 150 ug Cd/L. Karuppaswamy *et.al* (2005) found a significant decrease in total erythrocyte count, haaemoglobin content, haematocrit value and mean corpuscular haemoglobin concentration in air breathing fish, *Channa punctatus* after exposure to sub lethal dose of Cd (29 / ml) the decrease in RBCs

count may be attributed to haematopathology or acute haemolytic crisis that result in severe anemia in most vertebrates including fish species exposed to different environmental pollutants.

Annune *et.al.*, (1994b) observed a non-significant decrease in red cells of *Oreochromis niloticus*. The non-significant decrease in erythrocyte count and erythrocyte sedimentation rate of *Heteroclarius* sp. may be attributed to the swelling of red blood cells. Flos *et.al* (1987) reported that the swelling of red blood cells (erythrocytes) may be due to an increase in protein and carbon dioxide in the blood. According to Khangarot and Tripathi (1991), the decrease in RBCs count may be attributed to haematopathology or acute haemolytic crisis that result in severe anemia in most vertebrates including fish species exposed to different environmental pollutants or may be the decrease in RBCs may be attributed to reduction of growth and other food utilization parameters which result in severe anemia (James and Sampath, 1999). The decrease in the total erythrocyte count (TEC) may be due to the cytotoxic effect of heavy metallic compounds on the erythropoietic tissue. Such a disturbance in bone marrow leads to alteration of cell cycle and reduction in erythropoeisis (Nunes *et.al.*, 2001; Tariq, *et.al* 2007; Sharma *et.al.*, 2007). The decrease in RBCs count may be attributed to haematopathology or acute haemolytic crisis that result in severe anemia in most vertebrates including fish species exposed to different environmental pollutants.

The red blood cell count of *Clarias gariepinus* was reported to have increased significantly by Annune *et.al.*, (1994a) when the fish was subjected to Zinc treatment. They attributed the red blood cell elevation to blood cell reverse combined with cell shrinkage as a result of osmotic alterations of blood by the action of the metal (Tort and Torres, 1988).

According to the results obtained from the present study Hb seems to be the best blood indicator of environmental stress. This finding is in agreement with Saint –Paul (1984) and Cazenave *et.al* (2005). They suggested that the increase in Hb concentration could be an especially reliable first indicator of an adaptational improvement in the oxygen transporting capacity of the blood. In addition to

behavioral and morphological adjustments, fish could respond to low oxygen levels by adjusting several physiological and biochemical parameters (Val *et.al.*, 1998).

Reduction in haemoglobin concentration may probably be due to production of reactive oxygen species under the influence of heavy metals cadmium and lead, which results in destruction of the red blood cell membrane and its function (Tariq, 2007). The observed depiction in the haemoglobin and haematocrit values in the fish could also be attributed to the lysing of erythrocytes. Similar reductions have been reported by Sampras *et.al.* (1993), and Musa and Omoregie (1999) when they exposed fish to polluted environment under laboratory conditions. Thus, the significant reduction in these parameters is an indication of severe anemia caused by exposure of the experimental fish to cadmium in the water.

Typically, haematological parameters are non-specific in their responses towards chemical stressors. However, it is well known that toxic substances can significantly damage the haematological system of fish (Vander Oost *et.al.*, 2003). Some changes may be the result of a disorder in erythrocyte cell membrane permeability and /or the result of the activation of protective mechanisms. These mechanisms may include the release of erythrocytes from blood deposits and /or from haemapoitic tissues into the blood stream (Svodova *et.al.*, 1994). On the other hand the haematocrit gives an indication of the hemopoitic activity of the animal. Abnormal haematocrit also can indicate nutritional deficiencies, the presence of disease-causing micro-organisms, and other health aberrations (Blaxhall, 1972 and Anderson, 1990). Therefore haematology may provide important information on the general physiology and health status of the organisms living under environmental stress.

Flos *et.al.* (1987) observed an increase in haematocrit levels in different fish species after Zinc treatments. They attributed such an increase in haematocrit values to increase in the size of the erythrocytes as being demonstrated for chromium and zinc treated rainbow trout. Observed depression in haematocrit and haemoglobin values coupled with decreased and deformed erythrocytes are obvious signs of anemia. The fish muscle has been known as the water exchange tissue with blood. Mishra and Srivastava (1979, 1980), Neumosok and Hughes (1998) observed

haemoconcentration after copper exposure and haemodilution following Zinc exposure in *Colis fasciatus*. In the present study, the decrease in haematocrit following cadmium exposure in *Etroplus maculates* may be an indication of haemodilution. Tort and Torres (1998) reported decrease in haematocrit following 24 hour exposure of dog fish, *Scyhorhinus canicula* to cadmium contamination. They attributed this decrease to haemodilution.

The calculated blood indices MCV, MCH and MCHC have a particular importance in anemia diagnosis in most animals. (Coles, 1986). The perturbations in these blood indices (Increase MCV, decrease of MCH and MCHC) may be attributed to a defense against Cd toxicity through the stimulation of erythropoiesis or may be related to the decrease in RBCs, Hb and Hct due to the exaggerated disturbances that occurred in both metabolic and hemopoietic activities of fish exposed to sub lethal concentration of pollutants. In the values obtained in the haematological indices, no significant change was recorded in the mean corpuscular volume (MCV) and mean corpuscular haemoglobin content (MCHC) but there was significant change in the mean corpuscular haemoglobin (MCH). However, slight fluctuations were recorded in the MCV and MCHC when compared with the control. Spleen contractions after stress have been detected in fish (Abrahamsson and Nilsson, 1975). Cells released from the spleen, which is an erythropoietic organ would have lowered the MCV values. A similar observation was made for *Cyprinus carpio* after cadmium exposure (Koyama and Ozaki, 1984). The significant change in the MCH may be due to the reduction in cellular blood iron, resulting in reduced oxygen carrying capacity of blood and eventually stimulating erythropoiesis. (Hodson *et.al.*, 1978).

High MCV suggests that a hemo-dilution mechanism is operational; the mean corpuscular volume gives an indication of the status or size of the red blood cells and reflects an abnormal/ normal cell division during erythropoiesis. The increase in MCV may be attributed to the swelling of the erythrocytes resulting in macrocytic anaemia. Such an increase in erythrocyte size is generally considered a response against stress and would be a consequence of several factors like high PCO2, high lactate concentration or low PO2 in the blood, leading to a low ATP

concentration, which would increase the oxygen affinity of blood (Soivo and Nikinmaa, 1981).

Spleen contractions after stress have been detected in fish (Abrahamsson and Nilsson, 1975). Cells released from the spleen, which is an erythropoietic organ would have lowered the MCV values. A similar observation was made for *Cyprinus carpio* after cadmium exposure (Koyama and Ozaki, 1984). The significant change in the MCH may be due to the reduction in cellular blood iron, resulting in reduced oxygen carrying capacity of blood and eventually stimulating erythropoiesis (Hodson *et.al.*, 1978).

Haematological parameters are very sensitive to stress. Decrease in the values of Hb, RBC, Ht, MCV with an increase in the concentration of the refined oil recorded in *Clarias gariepinus*, (Omoregie, 1998, Gabriel *et.al.*, 2007) and *Oreochromis niloticus* (Gabriel *et.al.*, 2001). But Prasad *et.al* (1987) recorded a decrease in the values of all the parameters but a significant increase in the Ht values of the cat fish, *Heteropneustes fossilis* exposed to crude oil. Decrease in the values attributed to haemolysis resulting in haemodilution, a mechanism for diluting the concentration of the pollutant in the circulatory system (Smith *et.al.*, 1979). Erythropaenia recorded in the exposed fish may be accounted for by swelling of the erythrocytes (Annune and Ahuma, 1998), damages to haemopoietic tissues in the kidneys and aggregation of cells at the gills thereby causing a decrease in the number of circulating cells of stressed fish (Singh and Singh, 1982).

Enhancement in the total leucocyte count (TLC) following heavy metal exposure could be possible due to leucocytosis as leucocytosis an outcome of proliferation of haemopoietic cells. Leading to progressive infiltration in peripheral blood (Tariq, 2007; Sharma *et.al.*, 2007). Changes in WBC and differential count have been reported to play important roles in the assessment of the state of health of fishes, and leucopenia and leukocytosis has been reported in the fish, *Clarias gariepinus* under exposure to pathogens, heavy metals and chemotherapeutants (Van Vuren *et.al.*, 1994, Ezeri, 2001; Omoregie and Oyebanji, 2002). Leukopaenia and /or leukocytosis are thus a normal reaction to stressors or irritants such as Kerosene. Significant leucopenia was recorded in *Clarias gariepinus* exposed to the

150 and 300 m/g kerosene. Leukopaenia has been reported in *Heteropneustes fossilis* exposed to crude oil (Prasad *et.al.*, 1987) and *Clarias gariepinus* exposed to copper (Van Vuren *et.al.*, 1994), whereas leukocytosis was reported on *Clarias gariepinus* infected with bacteria, *Psudomonas fluorescens* (Ezeri, 2001), malachite green (Musa and Omoregie, 1999) and copper and lead (Annune and Ahuma, 1998). The increasing or decreasing numbers of white blood cells are a normal reaction to a chemical such as zinc and cadmium.(Kori-Siakpere *et.al.*, 2006,2008), demonstrating the effect of the immune system under toxic conditions. The decreased number of white cells (leucopenia) may be the result of bioconcentration of the test metal in the kidney and liver (Agrawal and Srivastava, 1980). Decreased number of white blood cells may also be related to an increased level of corticosteroid hormones, whose secretion is a non specific response to any environmental stresser (Iwama *et.al.*1976, Ellis, 1981).

The different levels of the toxicant may have exerted varying degree of stress on the defense mechanism of the exposed fish and hence the production of different amounts of WBC (Ellis, 1977).Besides, toxicants differ in potency and mode of actions and also fishes respond differently to different toxicants (Rice *et.al.*, 1977). Pollutants and other stressers cause changes in the subpopulations of leukocytes (Ellis, 1977; Musa and Omoregie, 19999; Gabriel *et.al.*, 2007). Erythropenia associated with hypochromasia, leukocytosis with increase in large lymphocytes, thrombocytosis and hypercoagulability of blood was observed in *Heteropneustes fossilis* exposed to mercury and zinc. (Banerjee, 1998). The conditions accompanying this state are polythermia and hypo and haemolytic anemia (Sieved, 1964). According to Garcriel *et.al*, 2007) polycythermia is a disease of unknown cause characterized by an increased production of red blood cells above normal values. In haemolytic anaemia the red cells break down or haemolysis at an early stage.

In fish, any infestation with any organism activates the cellular and humoral immune system. This is followed by changes in circulating antibodies and percentages and absolute number of the different WBC (Boon *et.al* 1980). Quality and quantity of leukocyte cells which are haematologic parameters are generally

used to determine immune reactions and diseases (Ekingen, 1988, Cagirgan, 1990).In the present study an increase in WBC and neutrophil quantities were accepted as a response of cellular immune system to pollution. It can be concluded from the studies of Ulukoy and Timur (1993), Palikova and Navratil (2001) Sahan and Cengiler (2002) that immune system of fish creates similar responses to unfavourable conditions.

The increase in WBC observed in the present study could be attributed to a stimulation of the immune system in response to tissue damage caused by cadmium chloride. Gill and Pant (1985) have reported that the stimulation of the immune system causes an increase in lymphocytes by an injury or tissue damage. An increase in lymphocyte number may be the compensatory response of lymphoid tissue to the destruction of circulating lymphocytes (Shah and Altindag, 2005). In the white blood cell count, a sharp decrease was observed in the percentage neutrophil and oesinophils. The decrease in oesinophil was found to be significant. Te reduction in the percentage neutrophils and oesinophils here are in agreement with the findings of Sharma and Gupta (1984) when juveniles of mud fish, *Clarias batrachus* exposed to carbon tetrachloride. Musa and Omorejie (1999) also reported a decrease in neutrophils of *Clarias gariepinus* (Burchell) exposed to malachite green. This was attributed to tissue damage.

The higher Percentage of polymorphonuclear leukocyte usually indicates increase in the immunological response (Blaxhall and Daisely, 1973) and can be connected with maintaining of immunological activity in stressed population. Moiseenko, (1998) reported that in *Coregonus lavaretus* an increased response of white blood cells (WBC) and inferred that the response depends on environment pollution. Similarly, Saravanan *et.al.*. (2003) observed increased RBC and WBC contents in fish from polluted environment. Thus the number of RBC and WBC may vary according to environmental influence. Some authors have reported a decrease in RBC numbers and hematocrit in infected fish (Rehulka, 2002; Martins *et.al.*, 2004b) and in fish exposed to pollutants (Silveira-Coffigny *et.al*, 2004., Simonato *et.al.*, 2007), while an increase in the number of leucocytes and

neutrophils have been reported in parasitized fish (Sopinska, 1985; Silva-Souza *et.al.*, 2000, Ghiraldelli *et.al.*, 2006b.)

Lead nitrate exposed *Etroplus maculates* display lower stamina. Pb decreases heme synthesis (Bolognani Fantin *et.al.*, 1989; Tabche *et.al.*, 1990) and erythrocyte concentrations (Dawson, 1930, 1933, 1935) in fishes with in a few days. Among the heavy metals, lead is one of the metals known to man since medieval times. It is a non essential element being released in to the media either terrestrial or aquatic and is causing several toxicological problems to aquatic animals and man. The natural water is continuously being contaminated by lead due to increase anthropogenic activity and industrial exploitation of metal (Chandravathi and Reddy, 1996). The harmful effects caused by lead include haematological, biochemical and physiological alterations in several aquatic species (Chandravathi and Reddy. 1996). Literature shows that changes in haematological indices of fish caused by heavy metals and their mixtures are different. They are predetermined both by the concentration of heavy metals in water and the time of exposure, and both these factors can be reversible and irreversible changes in the homeostatic system of fish.

The toxic effects of heavy metal on fish are multidirectional and manifested by numerous changes in the physiological and chemical processes of their body systems (Dimitova *et.al.*, 1994). Sublethal toxicity of lead to fish produces haematological and neurological effects (Hodson *etal.*, 1984). It is well known that lead causes early mortality of mature red blood cells and inhibition of haemoglobin formation through inhibition of erythrocyte alpha-amino levulinic acid dehydrogenase (ALAD-D). The result is anaemia at high lead exposures or compensating erythropoiesis at lower exposure (Hodson *et.al.*, 1984). In the light of the present study, the mean value of PCV treatment groups showed progressive decrease than the control groups. A decrease in the erythrocyte count or in the percent of haematocrit indicates the worsening of an organism state and developing anaemia (Adeyemo, 2007).

Haemoglobin concentrations reflect the supply of an organism with oxygen and the organism itself tries to maintain them as much stable as possible. In the

present study a decreasing trend in the haemoglobin concentration of the treatment group compared to control group This is in agreement with the study of Adeyamo (2009) in *Clarias gariepinus*.. A decrease in the concentration of haemoglobin in blood is usually caused by the effect of toxic metals on gills, as well as decrease in oxygen which also suggests anaemia or confirms the toxic impact of lead on *Etroplus maculates*. A non-dose dependent reduction in RBC level of the treatments was observed in *Clarias gariepinus* when exposed to sublethal concentrations of lead (Adeyemo, 2007). Haematological indices (RBC count, concentration of haemoglobin and haematocrit) have been reported to indicate secondary responses of an organism to irritants (Rogers *et.al.*, 2003). Who concluded after their research that mechanism of lead toxicityoccurs by ion regulatory disruption? The reduction in WBC count of the treatment groups that was observed agrees with the report that the release of epinephrine during stress causes a decrease of leucocyte count, which shows the weakening of the immune system.

The MCV, MCH and MCHC increased considerably in all treatments compared to the control (Figures..).However, the increase in MCV was significant ($P<0.05$) only in group C and the increase in MCH and MCHC recorded by the treatments was significant ($P<0.05$) only in group. This is in agreement with the work of Shah (2006) following a short term exposure of tench (*Tinca tinca*) to lead. These alterations were attributed to direct or feedback responses of structural damage to RBC membranes resulting in haemolysis and impairement in haemoglobin synthesis, stress-related release of RBCs from the spleen and hypoxia, induced by exposure to lead (Shah, 2006). In the present study it has been observed that, in the lead exposed fishes the number of RBCs and haemoglobin percentage deceased significantly from the normal values. However, the differential leucocyte counts were deviating significantly from normal values. The increase was observed in the number of lymphocytes and eosinophils while decrease was noticed in the number of monocytes and neutrophils. This is in agreement with the work of Shan (2006), Adeyemo *et.al.*, (2007) and Mastan *et.al* (2009).

Ruff *et.al* (1993) observed improved cognitive abilities in Pb-poisoned children treated with the Pb-chelating drug meso 2,3-dimercaptosuccinic acid

(DMSA). Mechanisms of action were not investigated in either study. Friedheim et.al (1978) described the efficacy of treating Pb poisoning with DMSA. Several workers investigating Pb redistribution during DMSA chelation therapy found that DMSA significantly reduced brain and bone Pb levels (Cory-Slechta, 1988; Jones *et.al.*, 1994; Tandon *et.al.*, 1994., although the mode of action at the cellular level is not clear. By decreasing levels of Pb in the primary storage site (bone) and in those tissues critical for controlling behaviour (brain), DMSA has the potential to affect changes in Pb induced behavioural dysfunction.

The fish muscle has been known as the water exchange tissue with blood. Haemoconcentration and haemodilution have been described in previous works. Mishra and Srivastava (1979, 1980), Neumosok and Hughes (1998) observed haemoconcentration after copper exposure and haemodilution following Zinc exposure in *Colis fasciatus*. In the present study, the decrease in haematocrit following cadmium exposure in *Etroplus maculates* may be an indication of haemodilution. Tort and Torres (1998) reported decrease in haematocrit following 24 hour exposure of dog fish, *Scyhorhinus canicula* to cadmium contamination. They attributed this decrease to haemodilution.

The observed depiction in the haemoglobin and haematocrit values in the fish could also be attributed to the lysing of erythrocytes. Similar reductions have been reported by Sampras *et.al.* (1993), and Musa and Omoregie (1999) when they exposed fish to polluted environment under laboratory conditions. Thus, the significant reduction in these parameters is an indication of severe anaemia caused by exposure of the experimental fish to cadmium in the water. Flos *et.al.* (1987) observed an increase in haematocrit levels in different fish species after Zinc treatments. They attributed such an increase in haematocrit values to increase in the size of the erythrocytes as being demonstrated for chromium and zinc treated rainbow trout. Observed depression in haematocrit and haemoglobin values coupled with decreased and deformed erythrocytes are obvious signs of anaemia.

The red blood cell count of *Clarias gariepinus* was reported to have increased significantly by Annune *et.al.*, (1994a) when the fish was subjected to Zinc treatment. They attributed the red blood cell elevation to blood cell reverse

combined with cell shrinkage as a result of osmotic alterations of blood by the action of the metal (Tort and Torres, 1988). Annune *et.al.*, (1994b) also observed a non-significant decrease in red cells of *Oreochromis niloticus*. The non-significant decrease in erythrocyte count of *Heteroclarius* sp. may be attributed to the swelling of red blood cells. Flos *et.al* (1987) reported that the swelling of red blood cells (erythrocytes) may be due to an increase in protein and carbon dioxide in the blood. Sampling procedures could also be as a result of hypoxia or stress that causes these changes.

In the values obtained in the haematological indices, no significant change was recorded in the mean corpuscular volume (MCV) and mean corpuscular haemoglobin content (MCHC) but there was significant change in the mean corpuscular haemoglobin (MCH). However, slight fluctuations were recorded in the MCV and MCHC when compared with the control. Spleen contractions after stress have been detected in fish (Abrahamsson and Nilsson, 1975). Cells released from the spleen, which is an erythropoietic organ would have lowered the MCV values. A similar observation was made for *Cyprinus carpio* after cadmium exposure (Koyama and Ozaki, 1984). The significant change in the MCH may be due to the reduction in cellular blood iron, resulting in reduced oxygen carrying capacity of blood and eventually stimulating erythropoiesis (Hodson *et.al.*, 1978).

Chapter 6

Heavy Metal induced changes in enzymatic parameters

HEAVY METAL INDUCED CHANGES IN ENZYMATIC PARAMETERS

6.1 INTRODUCTION

Fish are largely being used for the assessment of the quality of aquatic environment and as such can serve as bioindicators of environmental pollution (Dautremepuits.et.al 2004; Lopas et.al., 2001).

There are studies examining the effects of sub lethal chronic concentrations of heavy metals on fish and these studies aim at morphologic and biochemical variations in the organs of different species of fish (Wong and Wong., 2000; Zhou et.al., 2001; Cavas changes that appear to be closely related. To counteract any stress, energy reserves, which might otherwise be utilized for growth, and reproduction will have to be diverted towards enhanced synthesis of detoxifying ligands (metal binding proteins, granules) , or expended in order to maintain an elevated efflux of metal. Consequently, various enzymes related to energy metabolism alter their activity pattern depending on the nature of stress. Excess energy is required to carry out defensive behavioral responses that help animal to adapt and survive. This confers some confidence in quantifying metabolic changes in the energy parameters, and related enzyme activities as integrated markers of healthy physiological status.

The organisms developed a protective defensive against the deleterious effects of essential and inessential heavy metals and other xenobiotics that produce degenerative changes like oxidative stress in the body (Abou EL-Naga et.al., 2005; Filipovic nd Raspor, 2003). Fish use lipids rather than carbohydrates as the main energy source like other poikilothermic organisms (Henderson and Torcher, 1987). In all living organisms, lipids are transported throughout the circulation bound to proteins in specific macromolecular complexes called lipoproteins (Fried et.al., 1968). Lipoproteins can be classified according to hydrated density, ultracentrifugation floatation velocity, and electriophoretic mobility on agarose and

apolipoprotein and these classes are: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) (Babin and Vernier, 1989).

The response of fish to environmental challenges are in due course reflected as overall alteration in metabolism. The responses in the beginning reflected are reversible, but long-drawn-out exposure to environmental pollutants brings about enduring pathological changes in the physiology of fish. These alterations are echoed as loss of surviival skills, inhibition of growth (George and Nagel, 1990; Alvarez and Fuiman, 2005) reproductive dysfunction (Tyler et.al., 1998), and immune suppression (Fatima et.al. 2000, 2001).

Carbohydrates are the main source of energy in living systems for their metabolic processes. Carbohydrates are the central point of energy production because of its great mobility in the living systems, together with its capacity to get compartmentalized with in the cell and tissues. The mobility is provided by glucose and compartmentalization by glycogen. Proteins are the most abundant organic molecules of living system and form the basis of structure and function of life. Proteins have many different physiological functions. Proteins have many physiological functions. They are associated with enzymes, transport and regulation of metabolism, defense, structural elements and storage and hence represent an important biochemical constituent in fish blood. Carbohydrates are the primary and immediate source of energy. When the need arises for energy, glycogen is converted to glucose in the serum. Blood glucose concentration appears to be a sensitive and reliable indicator of stress in fish. Rise in glucose levels in blood and tissues can be used to indicate the toxicity of pollutants in the ambient environment (Elizoic et.al., 1987) didn't get

Proteins are mainly involved in the architecture of the cell. During chronic period of stress they are also a source of energy (Umminger, 1977). During stress condition, fish needs more energy to detoxify the toxins and to overcome stress. Since fish have a very little amount of carbohydrates, the next alternative source of energy is protein.

Heavy metals accumulated in the tissues of fish may catalyze reactions that generate reactive oxygen species (ROS) which may lead to environmental oxidative stress. Defensive mechanisms to counteract the impact of ROS are found in many mammalian species including aquatic animals such as fish. These systems include various antioxidant defense enzymes such as superoxide dismutases which catalyze the dismutation of superoxide radical to hydrogen peroxide, catalase acting on hydrogen peroxide, glutathione S-transferase family possessing detoxifying activities towards lipid hydroperoxides generated by organic pollutants such as heavy metals (Tjalkens et.al., 1998).

Metal interact with legends in proteins particularly, enzymes and may inhibit their biochemical and physiological activities (Passow et.al., 1961). Current accepted opinion of cadmium action as well as other metals is related mainly to their influence on protein molecules, particularly enzymes. They have strong affinity to bond with the aminoacid molecules of protein and may cause changes in enzyme structures. The most obvious consequences of these changes are the inhibition of enzymes.

Cadmium may induced oxidative damage in different tissues by enhancing peroxidation of membrane lipids in tissues and altering the antioxidant systems of the cells. The peroxidative damage to the cell membrane may cause njury to cellular components due to the interaction of metal ions with the cell organells. Cadmium depletes glutathione and protein bound sulfhydryl groups resulting in enhanced production of reactive oxygen species such as superoxide ions, hydroxyl radicals and hydrogen peroxides. The reactive oxygen species result in increased lipid peroxidation. Cadmium largely influences fsh protective systems. It induces C-reactive protein and metallothioneins (Paul et.al., 1998; De Smet et.al., 200).

Aspartate aminotransferases (AAT) and Alanine aminotransferases (ALAT) are functioning link between carbohydrate and protein metabolism. It has a wide distribution in both mammalian and fish tissues (Gaudet et.al., 1975, Eze, 1983) existing in two forms. Viz. M (mitochondrial) and S (cytoplasm). ALAT catalyzes the conversion of alanine to pyruvate by the transfer of the amino group to α-ketoglutarate there by converting it to glutamate.

Alanine + α-ketoglutarate → pyruvate + glutamate

AAT catalyzes the conversion of aspartate to oxaloacetate and the transfer of amino group to ketoglutarate gives glutamate.

Aspartate + α-ketoglutarate → oxaloacetate + glutamate

Transaminases are intracellular enzymes which exist only in small amounts in serum. When an organ or body tissue is injured, increased levels of AAT and ALAT is released into the blood. The enzyme may leak into the plasma following the damage or dysfunction of reservoir tissue. Hence the assay of transaminases has become an indispensable tool in the clinical determination of the pathological conditions of the reservoir tissues and organs. (La Due et.al., 1954). The greater the extent of tissue damage, the greater the quantity of AAT that is released. Liver is richly endowed with ALAT and AAT and alterations in the plasma level of these enzymes pinpoint liver dysfunction. Organisms, in response to carbohydrate depletion, use other substrates to obtain the energy needed for its maintenance through gluconeogenesis. Alanine and aspartate are precursor for gluconeogenesis, among amono acids, and the initial reaction is catalyzed by alanine aminotransferase (ALT), and aspartate amino transferase (AST). Amino b transferases, also called transaminases, constitute a group of enzymes that catalyzes the inter conversion of aminoacids in α-ketoacids by transferring amino groups. ALT is also known as glutamic pyruvate transaminase (GPT), and AST as Glutamic oxaloacetate transaminase (GOT). ALT and AST which serve as a strategic link between carbohydrate and protein metabolism, play as an essential group of enzymes in the gluconeogenesis pathway. Beyond this, aminotransferases are good indicators of tissue lesions. They are known to be altered during various physiological and pathological conditions making it a possible biomarker.

Alkaline and acid phosphatases are intrinsic plasma membrane enzymes found in almost all animals, plants and microbes. They catalyses the hydrolysis of various phosphate containing compounds and acts as transphosphorylases at acid and alkaline PHs. Alkaline phosphatase and acid phosphatase have activity in alkaline and acidic PHs respectively. Acid phosphatase is involved in a variety of metabolic processes such as membrane permeability, steroidgenesis, growth and

cell differentiation. Alkaline phosphatase is involved in membrane transport and is a good marker of stress, in living systems.

Quantitative assessment of enzymes is a reliable indicator of stress imposed on the organism by environmental pollutants such as heavy metals (Cheng, 1983a). Many physiological processes including activity of many lysosomal hydrolytic enzymes are inhibited by heavy metals even though these metals may also activate certain enzymes. Two important phosphatases are Acid Phosphatase (ACP) and Alkaline Phosphatase (ALP), both differing in their sub cellular distribution. ALP activity was found to be highly concentrated in plasma membrane enriched fraction, where as ACP is associated with lysosomes. These enzymes are involved in a variety of metabolic activities such as permeability, growth and cell differentiation, protein synthesis, absorption and transport of nutrients, gonadal maturation, and steroidogenesis (Ram and Sathyanesan, 1985).

ALP (EC 3.1.3.) is a polyfunctional enzyme, present in the plasma membrane of all cells. It hydrolyses a broad class of phosphomonoester substrates, and acts as a transphophorylase at alkaline PH 9. ALP activity has been reported to be sensitive to heavy metal pollutants (Regoli and Principato, 1995).

Lactate dehydrogenase (LDH) forms the center for a precisely balanced symmetry between catabolism and anabolism of carbohydrates (Everse and Kaplan,1973). The rise in LDH level is the result of cell damage. Enormously high LDH content in the serum can e due to tissue damage. Necrosis or trauma (Altman, 1974) A fish under stress preferentially meets its energy requirements through anaerobic condition.

The enzyme actylcholinesterase (AChE) which catalyses the hydrolysis of acetylcholine is ubiquitous in the animal kingdom (Massoulie et.al., 1993), Walker and Thomson, 1991). It is a well characterized enzyme in the vertebrates because of its critical catalytic function at the cholinergic synapses. The enzyme acetylcholinesterase (AChE) (EC.3.1.1.7) hydrolyzes the neurotransmitter acetylcholine to acetate and choline at the cholinergic synapses, terminating nerve impulse transmission. AChE as a potential cell membrane marker enzyme is already approved (Mitchell, et.al., 1965; Severson et.al., 1972, Steck., 1974; Watts et.al.,

1978). Acetyl cholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are the two enzymes that hydrolyze the neurotransmitter acetylcholine to acetic acid and choline (Chuiko. 2000). Several studies have shown that high levels of AChE inhibition are needed to cause significant mortality in aquatic species, for both acute and chronic exposures (Van der Wel and Welling, 1989; Zinkl et.al., 1991; Ansari and Kumar, 1984).

In this chapter an attempt has been made to study the effect of heavy metal cadmium and lead on some of the enzymatic and biochemical parameters of orange chromide , Etroplus maculates.

6.2 MATERIALS AND METHODS

Blood was taken by Nonheparanized syringe was used for biochemical analysis. For biochemical analysis the blood samples were transferred from syringe to tubes and plasma was obtained after centrifugation at 4000 rpm for 10 min at 4 0C. Biochemical analysis was made by means of an automated system (Olympus ®AU 2700).

Plasma was obtained by centrifugation of blood at 3000 rpm for 15 min and nonhaemolysed plasma was stored in deep freezer for further biochemical analyses. After decapitation of fish, samples of liver and muscle were taken and frozen for further biochemical analyses. Plasma glucose was determined , using glucose kits supplied by Boehring Mannheium kit, according to Trinder (1969). Total protein content was determined colorimetrically according to Henry (1964) . Total lipids contents were determined colorimetrically according to Joseph et.al (1972). Activities of aspartate amninotransferase (AST) and alanine aminotransferase (ALT) were determined colorimetrically according to Reitman and Frankel (1957), while alkaline phosphatase (ALP) was measured by using Diamond diagnostics kits according to the method of Rec (1972). Also acid phosphatase (ACP) activity was determined according to the method of King and King (1954) Acetycholinesterase – ACHE

6.2.1 Assay of tissue and serum acetycholinesterse enzyme

Acetycholinesterase activity in liver, brain, gill and serum was estimated as per the method of Ellman et al(1961).

Reagents

Tris – HCl buffer (pH 7.4), 2.6 mM, acetylthiocholine iodide, 0.5mM DTNB (Ellman's reagent).

Procedure

Brain, liver and gill tissues of both test and control fish were dissected out and were homofenized with 1.5 volume of buffer (tris – HCl 50 mM, KCl 0.15 M, pH 7.4). The homogenate was centrifuged in a refrigerated centrifuge at 10000rpm for 20 mimutes at 4^0C. AChE activity was determined using the Ellman's reagent DTNB (5,5" – dithio – bis (2-nitrobenzonic acid); 0.5mM) and acetylthiochiline iodide as substrate (Ellman et al. 1961). 50µl of the supernatant/serum was taken for assay, and 2.3 ml of 0.5 mM DTNB was added to it. 100 µl of 2.6mM Ach was added. The rate of change of absorbance was measured at 412nm. Blanks samples were taken to make sure that there was no non-specific esterase or other background activity. Protein was estimated as described by Lowry et al., (1951) allowing the calculation of AChE as U (mmol/min) /mg protein.

6.2.2 Estimation of tissue and serum acid phosphatase activity (ACP)

Both serum and tissue phosphatase activity was determined following the method introduced by Anon, 1963.

Regents

Pnitro phenyl phosphate, 0.1 N NaOH, 0.1 M. phosphate buffer.

Procedure

Liver, brain and gill of both control and test fishes were homogenized in isotomic sucrose and were centrifuged at 5000 rpm for 15 minutes. Supernatant obtained was the source of enzymes. 0.5ml of p-nitrophenyl phosphate was mixed with equal volume of 0.1 M phosphate buffer (pH 4.8). The enzyme was added and incubated for 30 minutes at room temperature. The reaction was arrested by adding

4ml of 0.1 N NaOH. The absorbance of solution was measured spectrophotometrically at 410nm. The amount of p-nitro phenol liberated by the acid phosphatase per hour per mg protein gives the specific activity. Protein was determined as per the method of Lowry et al (1951).

6.2.3 Estimation of tissue and serum alkaline phosphate (ALP) activity

Both serum and tissue phosphatase activity was determined following the method introduced by king (1965).

Procedure

1.5ml of carbonate-bicarbonate buffer, 1.0ml of substrate and 0.1 ml of magnesium chloride and requiste amount of the enzyme source were mixed together. The reaction mixture was incubated at 37°C for 15 minutes. The reaction was terminated by adding 0.1ml of folin's phenol reagent. Controls were incubated with out adding enzyme source and enzyme source were added after the addition of folin's phenol reagent. 1ml of 15% sodium carbonate solution was added and incubated for a further 10 minutes at 37°C. The blue colour developed was read at 640 nm against a blank. Standards also were treated similarly. The enzyme activity was expressed as micromoles of phenollberated/ mg protein/hr.

Estimation of tissue lactate dehydrogenase (LDH) Activity

Lactate dehydragenase activity was measured according to the method of Bergmeyer and bernt 1974.

Reagents

50 mM phosphate buffer (pH 4.50, 0.6 mM pyruvate , 0.18mM NADH.

Statistical analysis

Data were statistically analyzed by three factors ANOVA, followed by LSD analysis using statistical software SPSS-16.

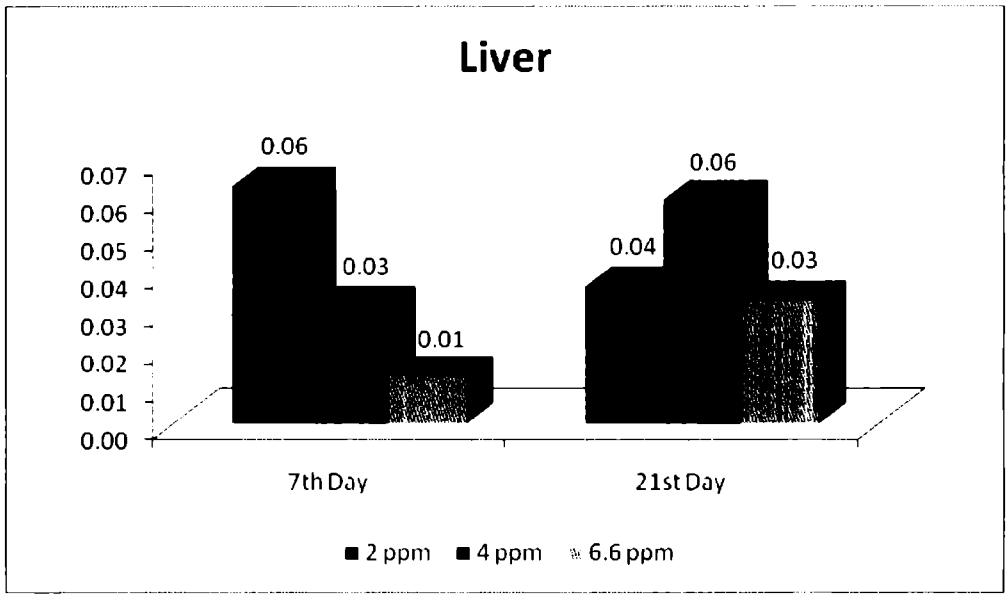
6.3 RESULTS

Acetylcholinesterase activities of the heavy metal treated fish were significantly decreased. The AChE activity in the brain, gill and liver showed a continues decrease as the exposure progressed. Maximum decrease was observe at the highest concentration of Heavy metal at 21 days exposure. Decrement of the enzyme activity was more intense as the time of exposure increased.

Table 6.1

Effect of cadmium on the Acetyl cholinesterase activity on the tissues of *Etroplus maculatus*

Tissue and serum	Control	0.33ppm		0.67ppm		1.2ppm	
		7th	21st	7th	21st	7th	21st
Liver	0.1023	0.0504	0.0394	0.0303	0.0210	0.0184	0.0111
	0.1087	0.0626	0.0320	0.0313	0.0229	0.004	0.0199
	0.1032	0.0584	0.0383	0.0308	0.0294	0.0173	0.124
	0.1038	0.0783	0.0312	0.0314	0.2252	0.0164	0.0114
	0.1094	0.0689	0.0394	0.0318	0.0294	0.0094	0.0184
	0.1028	0.0594	0.0386	0.0324	0.0286	0.0104	0.0118
Brain	0.3538	0.1098	0.0994	0.0909	0.0730	0.0623	0.0446
	0.3624	0.1114	0.0998	0.0914	0.0728	0.0684	0.0426
	0.3628	0.1118	0.0989	0.0924	0.0718	0.0694	0.0486
	0.3594	0.1098	0.0995	0.0908	0.0718	0.0694	0.0486
	0.3621	0.1113	0.0921	0.0918	0.0713	0.0676	0.0418
	0.3727	0.1124	0.0991	0.0912	0.0756	0.0634	0.0428
Gill	0.1978	0.1008	0.0804	0.0458	0.0286	0.0214	0.0142
	0.1924	0.1006	0.0801	0.0424	0.0293	0.0218	0.0146
	0.1973	0.1002	0.0812	0.0434	0.0286	0.0221	0.0152
	0.1989	0.1005	0.0807	0.0453	0.0289	0.0218	0.0142
	0.1934	0.1007	0.0817	0.0428	0.0291	0.0223	0.0154
	0.1971	0.1007	0.0805	0.0421	0.0298	0.0228	0.0164
Serum	0.2628	0.2019	0.1294	0.1471	0.0781	0.1087	0.0392
	0.2624	0.2014	0.1286	0.1469	0.0783	0.1084	0.0391
	0.2627	0.2017	0.1295	0.1468	0.0779	0.1079	0.0390
	0.2628	0.2018	0.1272	0.1459	0.0764	0.1082	0.0388
	0.2623	0.2017	0.1271	0.1465	0.0764	0.1079	0.0395
	0.2629	0.2018	0.1275	0.1469	0.0761	0.1078	0.0391


Liver
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0.03
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7th Day
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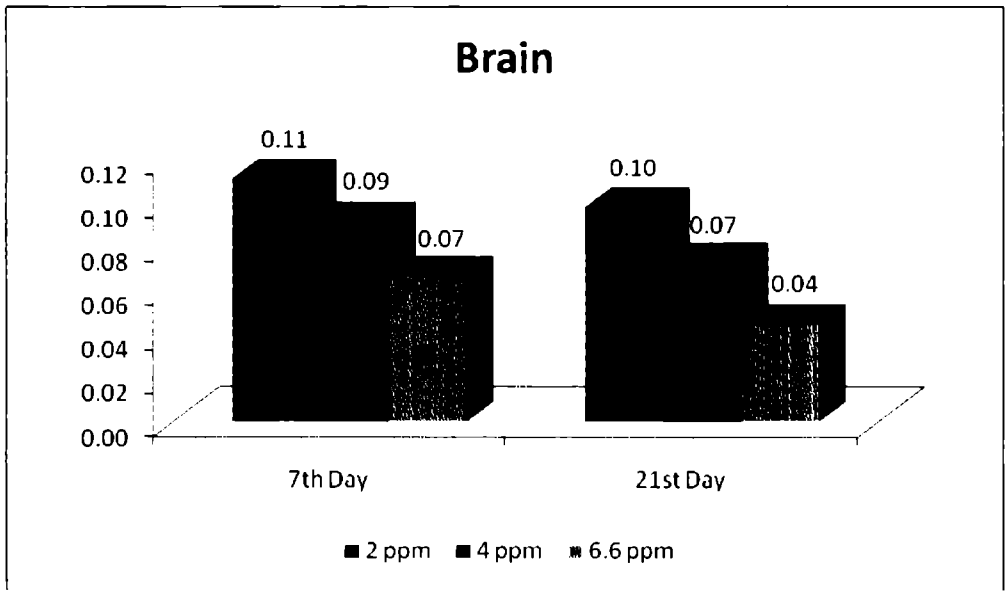
2 ppm
4 ppm
6.6 ppm


Brain
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0.09
0.07
0.10
0.07
0.04
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0.08
0.06
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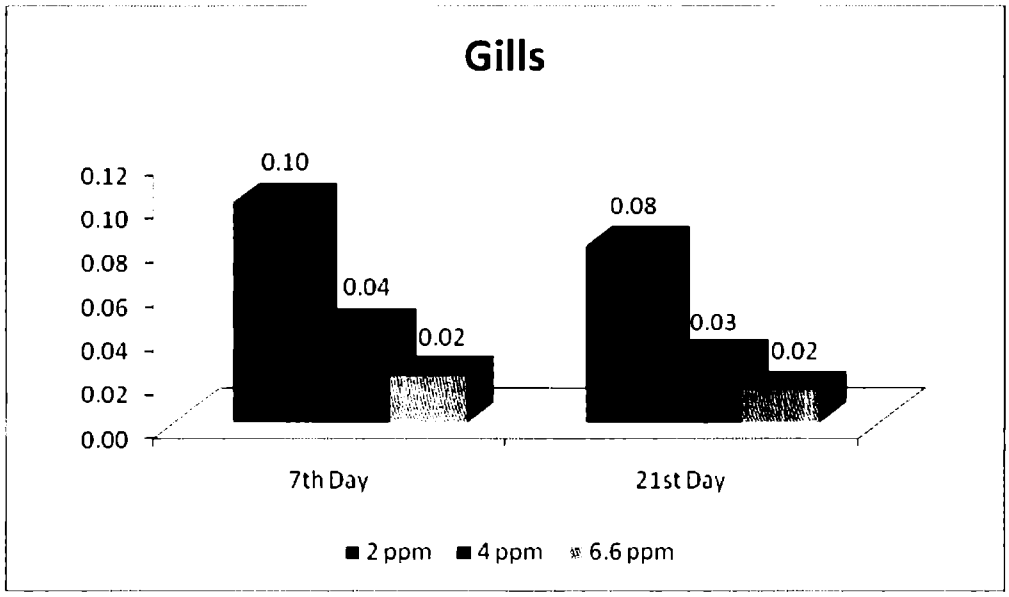
0.00
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Gills
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0.10
0.08
0.06
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7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

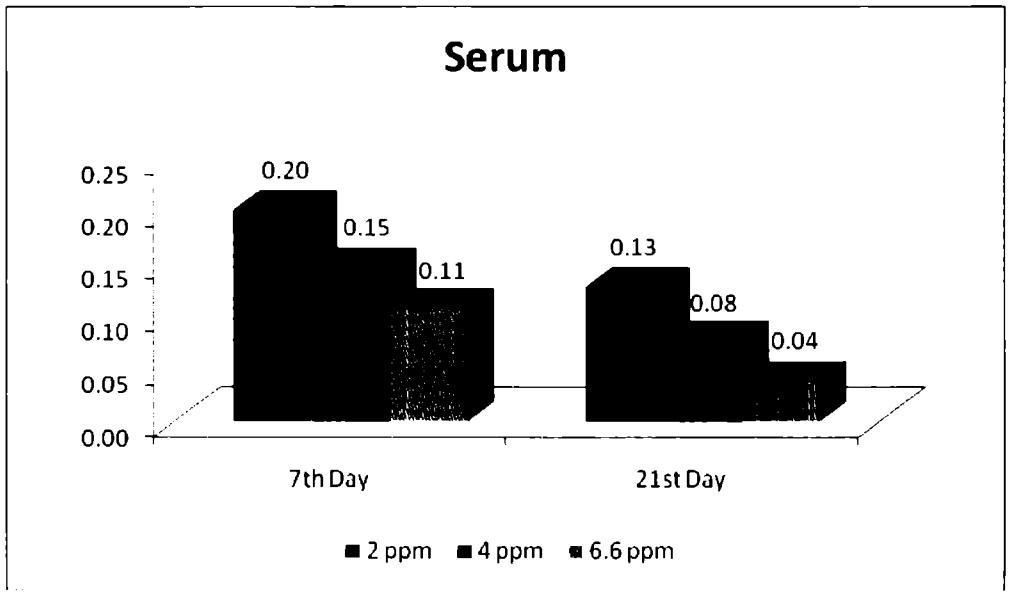
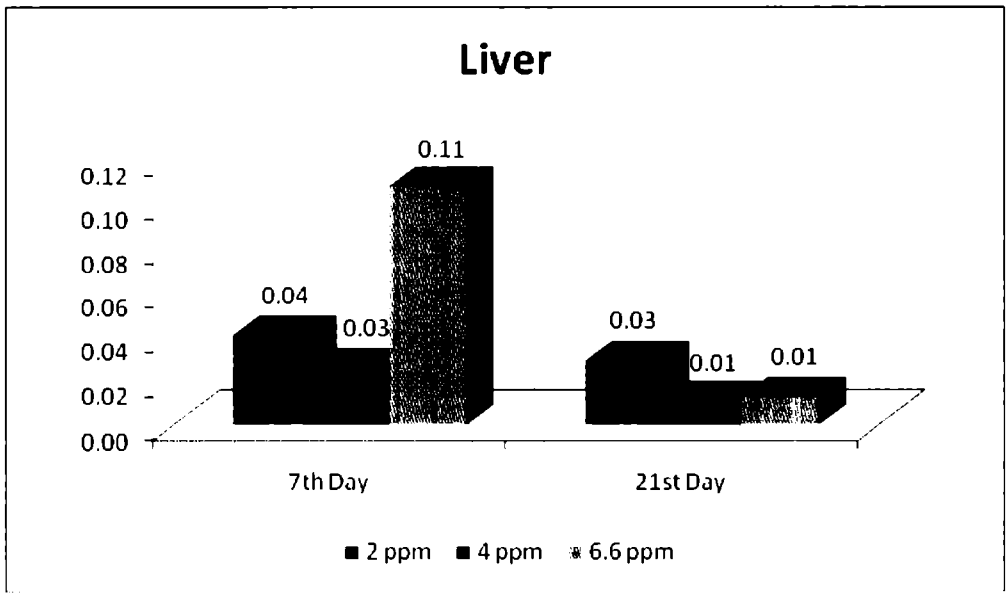
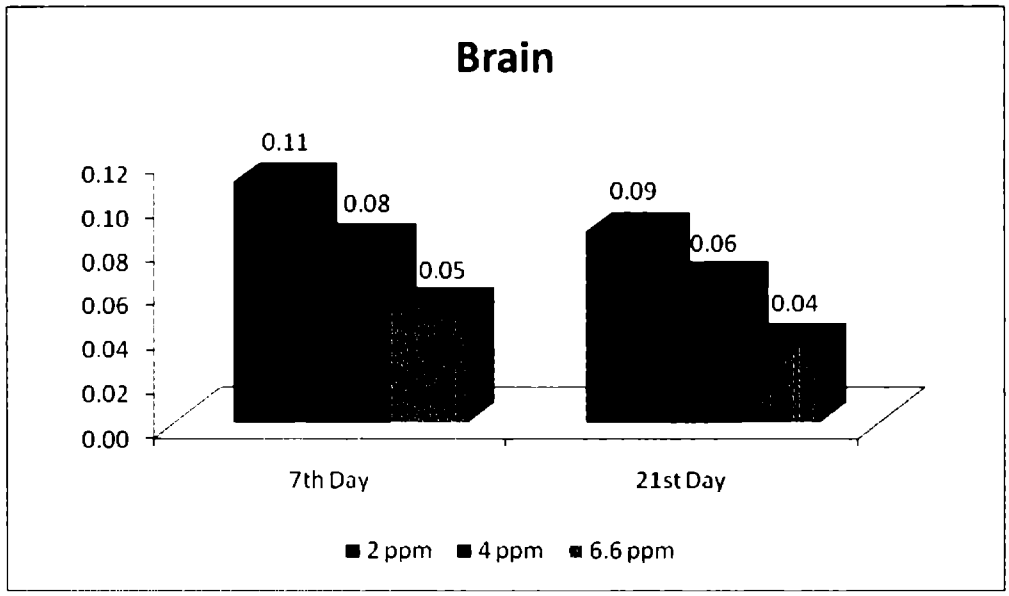

Serum
0.25
0.20
0.15
0.10
0.05
0.00
0.20
0.15
0.11
0.13
0.08
0.04
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

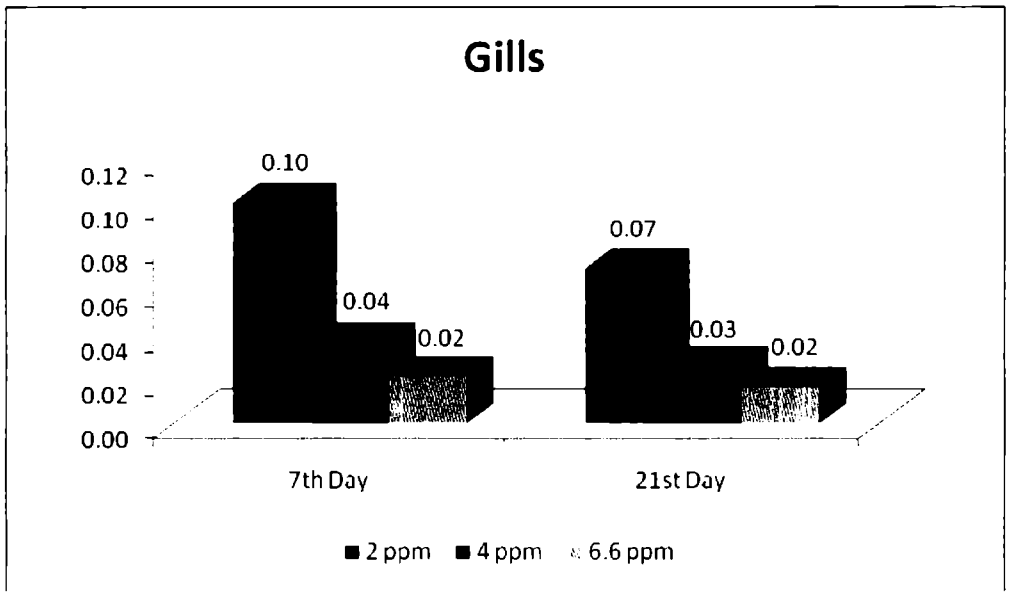
Table 6.2

Effect of lead on the Acetyl cholinesterase activity on the tissues of *Etroplus maculatus*

Tissue and serum	Control	2		4		6.6	
		7^{th}	21^{st}	7^{th}	21^{st}	7^{th}	21^{st}
Liver	0.1003	0.0403	0.0296	0.0236	0.0113	0.0124	0.0093
	0.1003	0.0402	0.0291	0.0255	0.0112	0.129	0.0101
	0.1003	0.0404	0.0293	0.027	0.0114	0.128	0.0094
	0.1002	0.0401	0.0289	0.025	0.0104	0.127	0.0191
	0.1004	0.0405	0.0289	0.027	0.0105	0.126	0.0191
	0.1002	0.0402	0.0271	0.025	0.0102	0.127	0.0094
Brain	0.3536	0.1095	0.0894	0.0810	0.0642	0.0523	0.0329
	0.3537	0.1092	0.0844	0.0812	0.0652	0.0549	0.0391
	0.3531	0.1091	0.089	0.0819	0.0614	0.0521	0.0331
	0.3532	0.1099	0.0879	0.0816	0.0653	0.0524	0.0394
	0.3529	0.1094	0.0827	0.0829	0.0649	0.0522	0.034.
	0.3529	0.1084	0.0879	0.0814	0.0652	0.0521	0.0375
Gill	0.1978	0.1007	0.0705	0.0360	0.0267	0.0204	0.0131
	0.1979	0.1006	0.0715	0.0343	0.0252	0.0204	0.0131
	0.1971	0.1005	0.0691	0.0354	0.0268	0.029	0.034
	0.1978	0.1005	0.0705	0.0360	0.0267	0.0204	0.0138
	0.1978	0.1005	0.0705	0.0360	0.0267	0.0205	0.0131
	0.1971	0.1006	0.0715	0.0462	0.0268	0.0215	0.0152
Serum	0.2521	0.2018	1.286	0.1473	0.0683	0.1081	0.0371
	0.2524	0.2017	0.1296	0.1473	0.0684	0.1084	0.0399
	0.2524	0.2018	0.1276	0.1471	0.0681	0.1094	0.0371
	0.2524	0.2012	0.1271	0.1477	0.0687	0.1097	0.0372
	0.2521	0.2014	0.1273	0.1475	0.0685	0.100	0.0291
	0.2528	0.2015	0.1271	0.1473	0.6931	0.102	0.0291


Liver
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.04
0.03
0.11
0.03
0.01
0.01
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Brain
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.11
0.08
0.05
0.09
0.06
0.04
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Gills
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.10
0.04
0.02
0.07
0.03
0.02
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

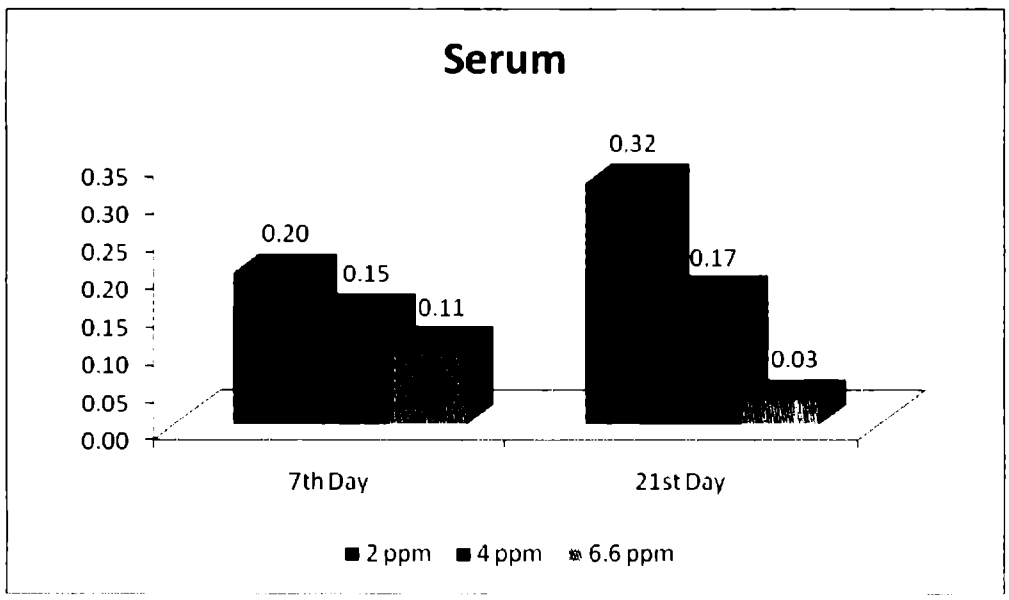
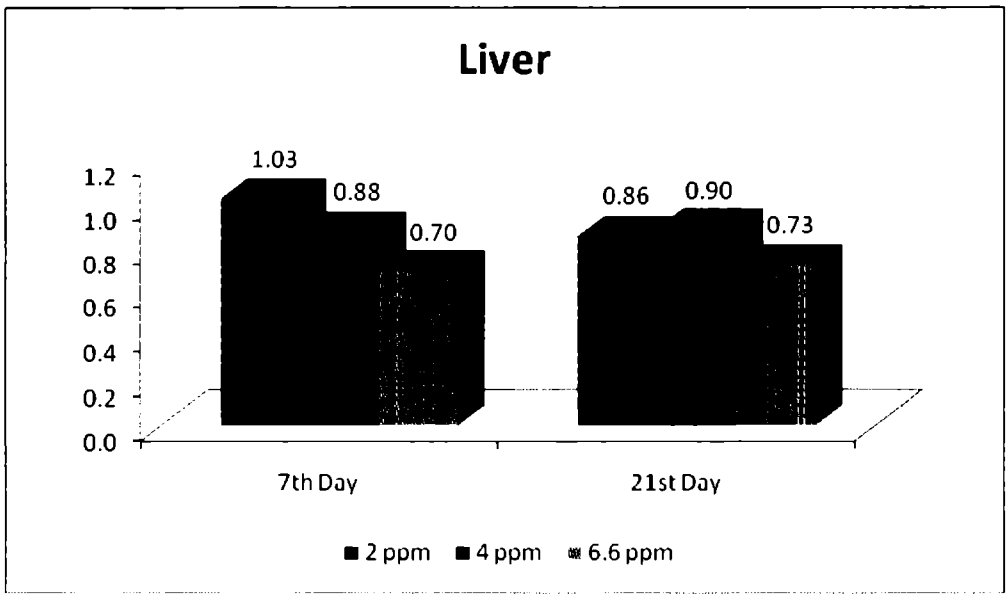
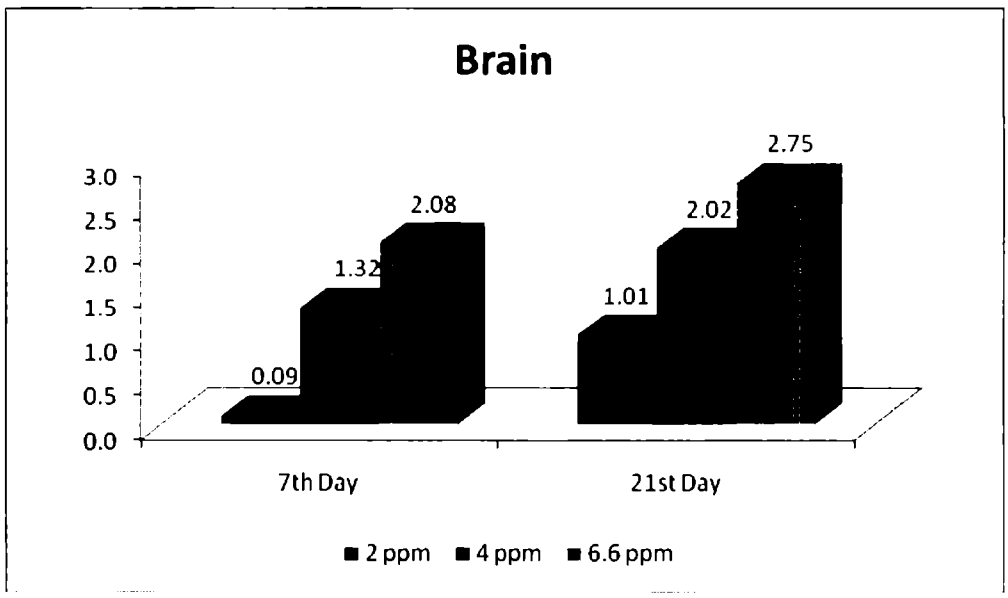

Serum
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.20
0.15
0.11
0.32
0.17
0.03
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

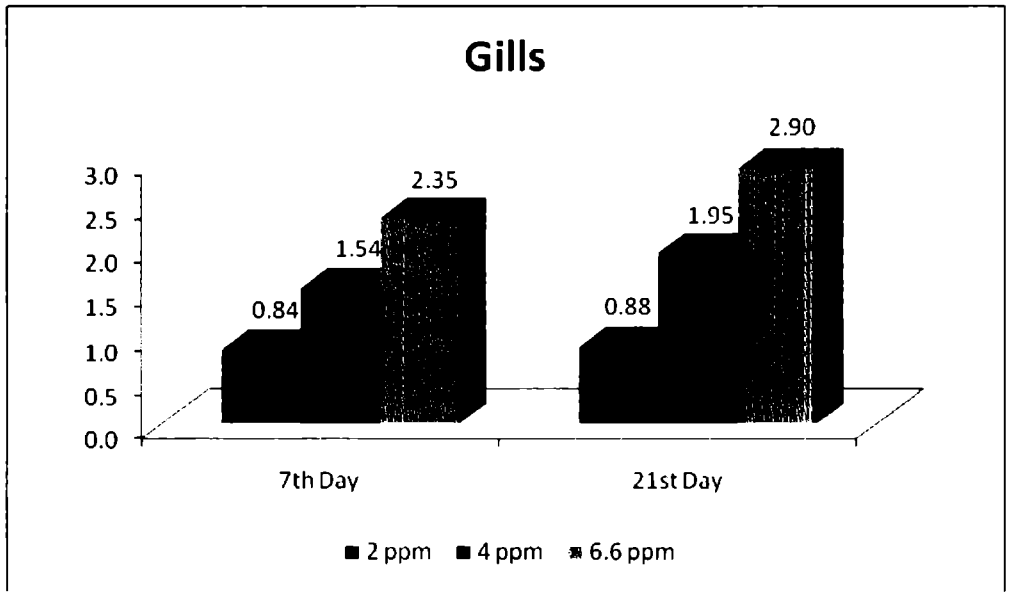
Table 6.3

Effect of cadmium on the alkaline phosphatase activity on the tissues of *Etroplus maculatus*

Tissue and serum	Control	0.33ppm		0.67ppm		1.2ppm	
		7^{th}	21^{st}	7^{th}	21^{st}	7^{th}	21^{st}
Liver	1.08	1.02	0.86	0.88	0.89	0.70	0.72
	1.08	1.03	0.85	0.88	0.89	0.71	0.73
	1.08	1.038	0.851	0.889	0.898	0.70	0.73
	1.07	1.02	0.86	0.88	0.897	0.709	0.731
	1.079	1.029	0.86	0.871	0.898	0.701	0.741
	1.081	1.031	0.871	0.872	0.897	0.709	0.738
Brain	0.919	0.038	1.019	1.321	2.01	2.07	2.79
	0.918	0.036	1.012	1.301	2.021	2.071	2.79
	0.916	0.037	1.013	1.341	2.03	2.081	2.78
	0.918	0.038	1.014	1.328	2.02	2.089	2.69
	0.917	0.038	1.015	1.319	2.01	2.087	2.699
	0.916	0.336	1.015	1.329	2.02	2.079	2.78
Gill	0.75	0.86	0.88	1.57	1.93	2.33	2.93
	0.74	0.87	0.89	1.56	1.91	2.35	2.97
	0.72	0.83	0.86	1.52	1.94	2.36	2.91
	0.73	0.81	0.87	1.54	1.98	2.34	2.89
	0.76	0.84	0.88	1.53	1.97	2.36	2.78
	0.75	0.85	0.87	1.52	1.96	2.35	2.93
Serum	4.56	5.07	5.69	4.85	5.94	6.00	6.84
	4.571	5.098	5.698	4.879	5.891	6.010	6.845
	4.578	5.098	5.671	4.869	5.871	6.018	6.852
	4.596	5.099	5.673	4.719	5.933	6.019	6.861
	4.569	5.099	5.671	4.731	5.871	6.012	6.791
	4.593	5.0181	5.613	4.853	5.813	6.019	6.781


Liver
1.03
0.88
0.70
0.86
0.90
0.73
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Brain
2.75
2.08
2.02
1.32
1.01
0.09
3.0
2.5
2.0
1.5
1.0
0.5
0.0
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Gills
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.84
1.54
2.35
0.88
1.95
2.90
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

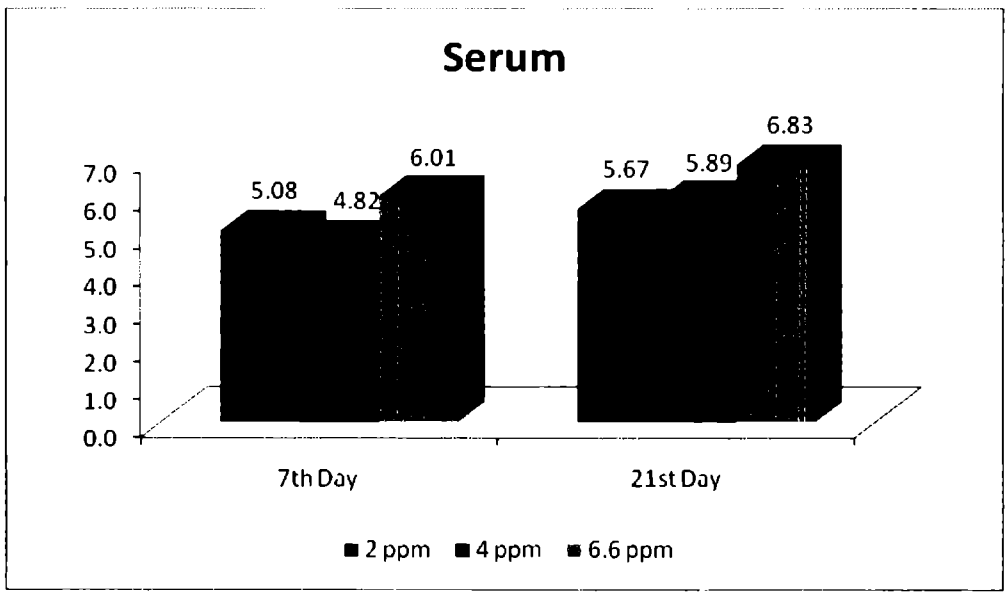
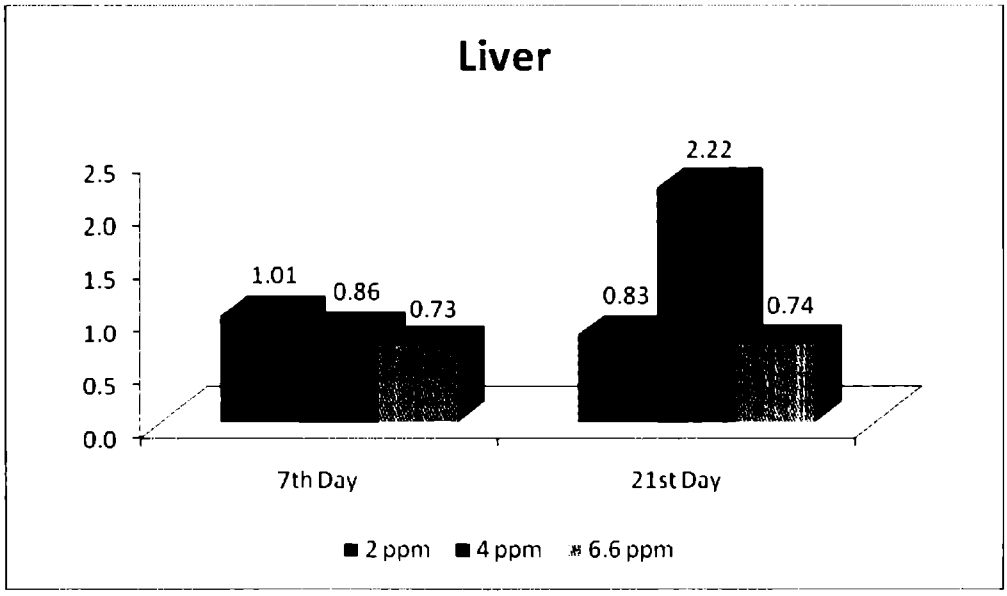
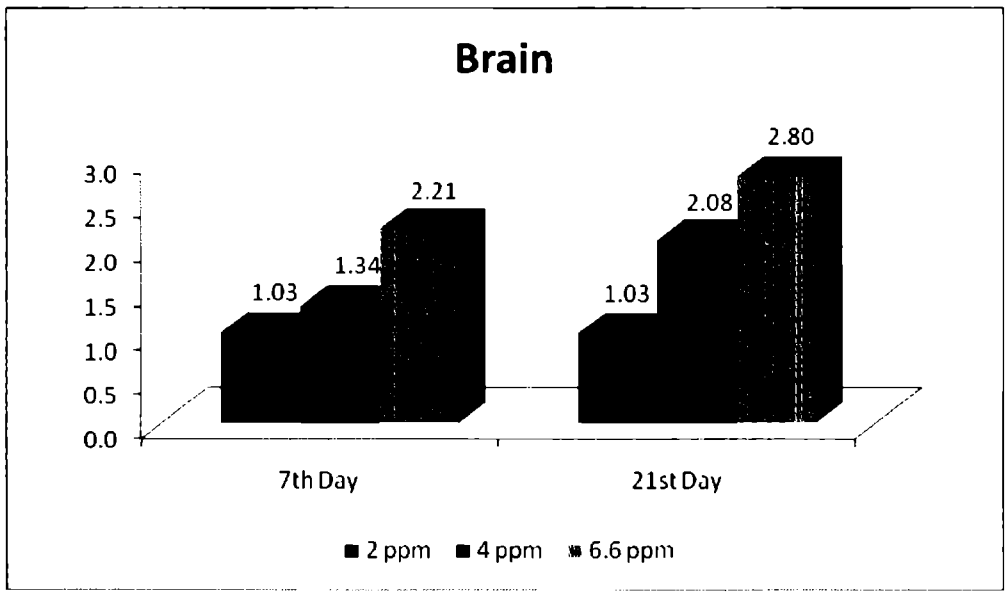

Serum
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
5.08
4.82
6.01
5.67
5.89
6.83
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

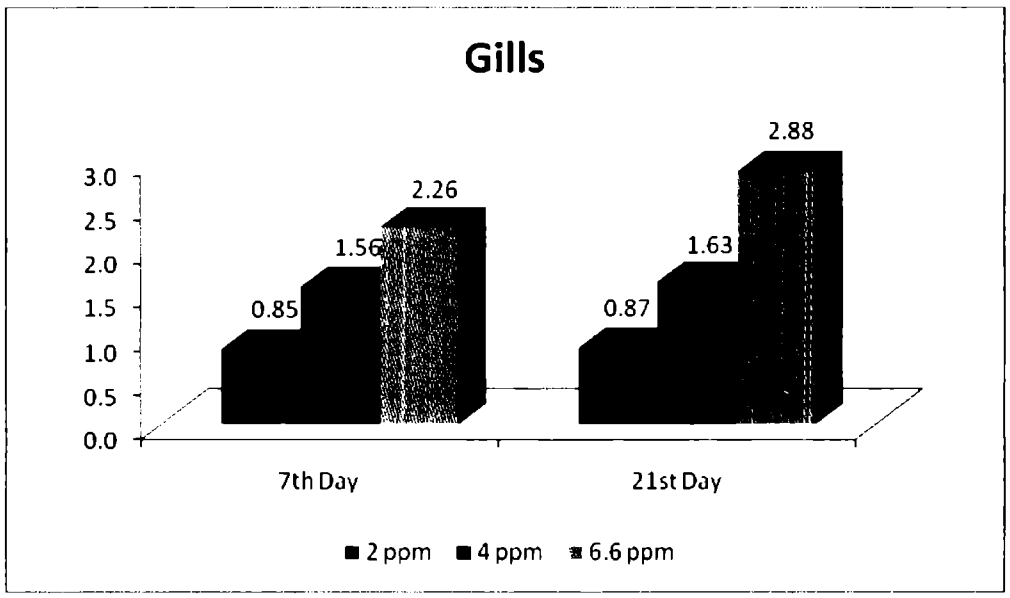
Table 6.4

Effect of lead on the alkaline phosphatase activity on the tissues of *Etroplus maculatus*

Tissue and serum	Control	2		4		6.6	
		7^{th}	21^{st}	7^{th}	21^{st}	7^{th}	21^{st}
Liver	1.079	1.018	0.84	0.87	0.899	0.718	0.72
	1.076	1.018	0.84	0.86	8.88	0.71	0.74
	1.075	1.012	0.79	0.85	0.88	0.799	0.75
	1.08	1.013	0.82	0.87	0.88	0.712	0.74
	1.081	1.014	0.83	0.86	0.88	0.719	0.745
	1.081	1.012	0.85	0.87	0.89	0.721	0.75
Brain	0.90	1.02	1.01	1.34	2.08	2.16	2.8
	0.89	1.03	1.013	1.35	2.09	2.17	2.81
	0.91	1.04	1.09	1.34	2.044	2.14	2.85
	0.90	1.03	1.019	1.351	2.098	2.17	2.679
	0.91	1.04	1.019	1.34	2.084	2.27	2.789
	0.89	1.04	1.013	1.289	2.094	2.35	2.881
Gill	0.75	0.89	0.89	1.56	1.94	2.32	2.90
	0.75	0.84	0.87	1.55	1.55	2.24	2.87
	0.75	0.84	0.85	1.57	1.57	2.25	2.87
	0.75	0.84	0.86	1.56	1.56	2.25	2.90
	0.76	0.85	0.86	1.57	1.57	2.24	2.87
	0.75	0.83	0.86	1.56	1.56	2.23	2.86
Serum	4.56	5.08	5.71	4.83	5.91	5.08	6.94
	4.568	5.07	5.69	4.79	5.90	6.01	6.97
	4.571	5.09	5.68	4.78	5.89	6.07	6.93
	4.573	5.093	5.69	4.71	5.91	6.075	6.94
	4.56	5.095	5.61	4.81	5.93	6.09	6.89
	4.579	5.089	5.72	4.84	5.94	6.081	6.935


Liver
2.5
2.0
1.5
1.0
0.5
0.0
1.01
0.86
0.73
0.83
2.22
0.74
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Brain
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.03
1.34
2.21
1.03
2.08
2.80
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Gills
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.85
1.56
2.26
0.87
1.63
2.88
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

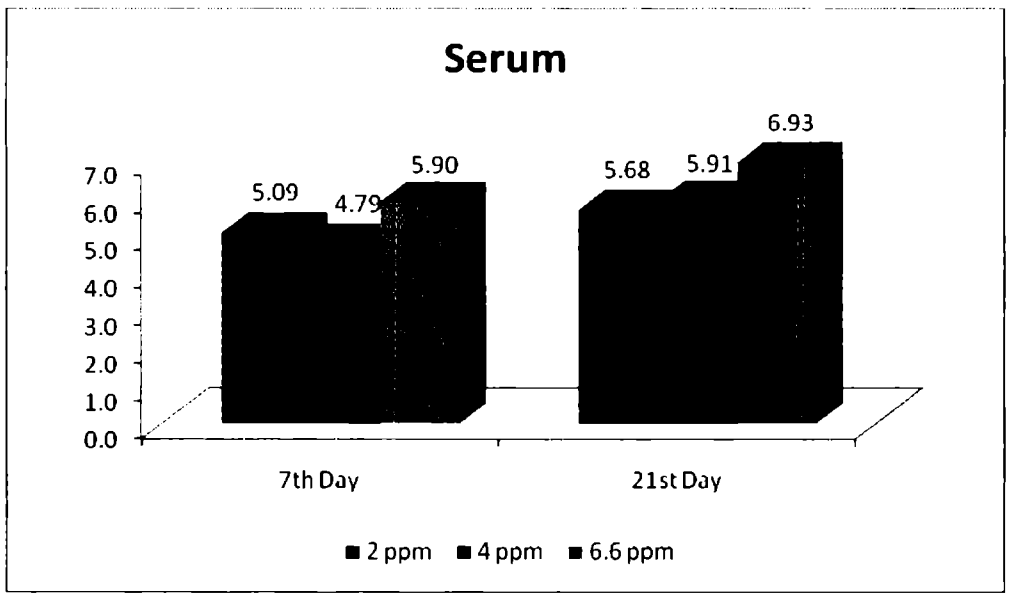
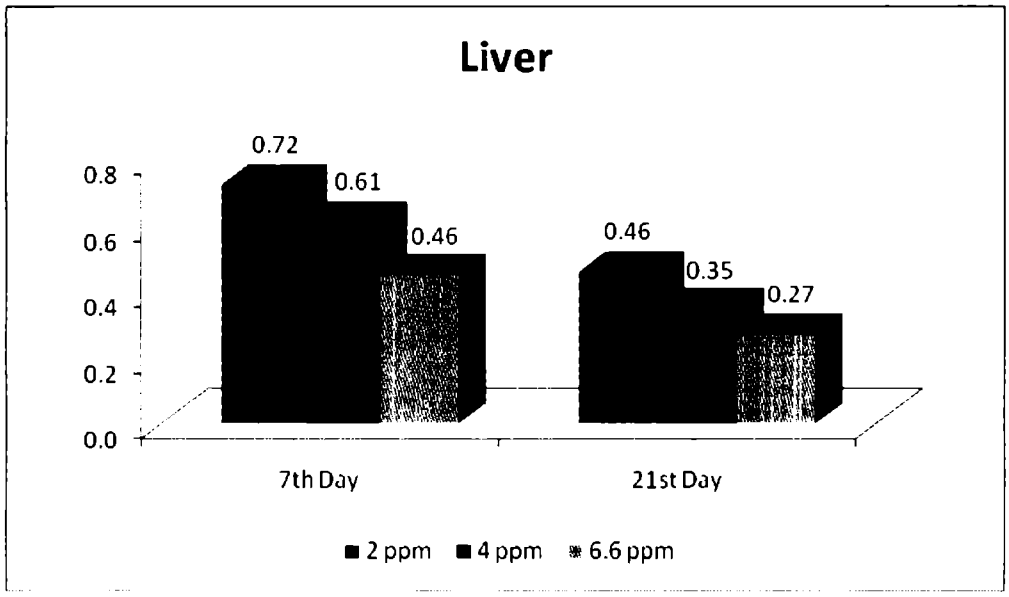
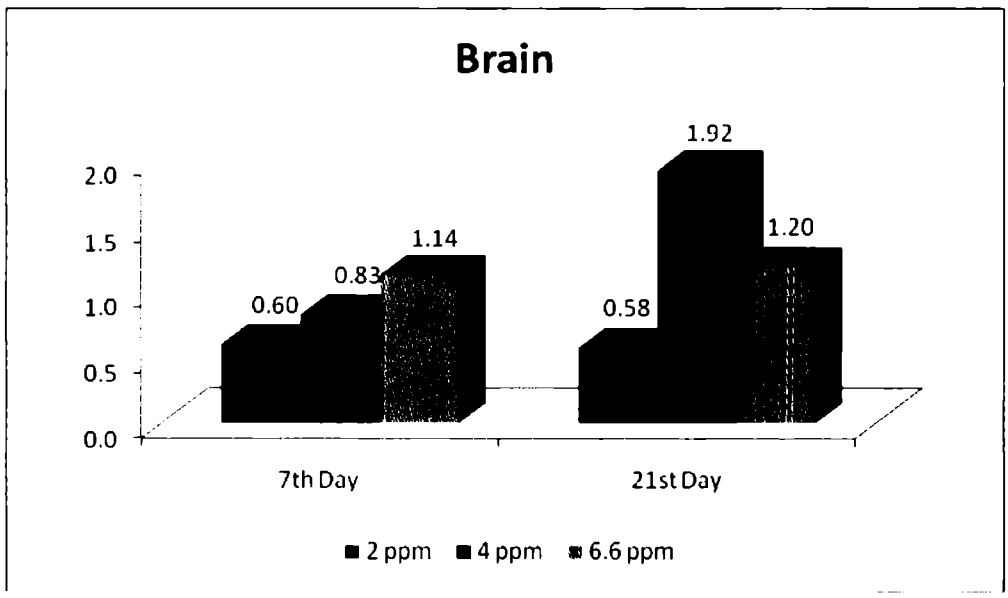

Serum
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
5.09
4.79
5.90
5.68
5.91
6.93
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

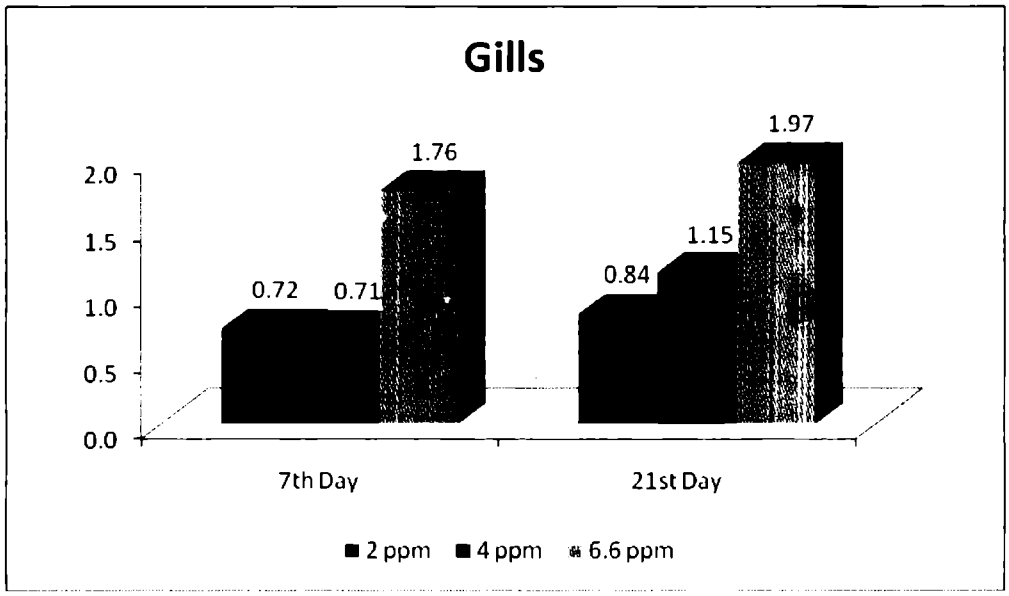
Table 6.5

Effect of lead on the acid phosphatase activity on the tissues of *Etroplus maculatus*

Tissue and serum	Control	2		4		6.6	
		7th	21st	7th	21st	7th	21st
Liver	0.986	0.707	0.4624	0.621	0.3517	0.467	0.2783
	0.987	0.701	0.4624	0.619	0.3527	0.452	0.2782
	0.976	0.789	0.4618	0.611	0.3489	0.451	0.2693
	0.981	0.706	0.4601	0.601	0.3491	0.451	0.2718
	0.982	0.716	0.4624	0.603	0.3591	0.451	0.2717
	0.985	0.713	0.4613	0.613	0.3516	0.461	0.2713
Brain	0.5884	0.6013	0.0769	0.8373	1.0914	1.1189	1.1329
	0.5884	0.6014	0.6761	0.8273	1.0814	1.124	1.1546
	0.5854	0.6012	0.6611	0.8271	1.0816	1.134	1.1546
	0.5881	0.6013	0.6761	0.8273	1.0812	1.132	1.1525
	0.5881	0.6013	0.679	0.8191	1.0887	1.121	1.415
	0.5871	0.6012	0.681	0.8213	6.079	1.181	1.181
Gill	0.6307	0.7145	0.8325	0.7008	1.1512	1.721	1.941
	0.6312	0.7146	0.8365	0.709	1.1491	1.719	1.984
	0.6312	0.7246	0.8395	0.724	1.1341	1.781	1.991
	0.6314	0.7186	0.8364	0.709	1.1512	1.781	1.989
	0.6294	0.7286	0.8352	0.708	1.1542	1.785	1.974
	0.6298	0.7249	0.8362	0.703	1.1513	1.795	1.941
Serum	4.012	4.203	4.819	4.89	5.1179	5.17	5.841
	4.012	4.203	4.814	4.891	5.1078	5.169	5.855
	4.011	4.201	4.817	4.871	5.1067	5.179	5.854
	4.012	4.200	4.814	4.891	5.1181	5.199	5.914
	4.012	4.203	4.817	4.871	5.1067	5.179	5.854
	4.012	4.203	4.819	4.89	5.117	5.17	5.98


Liver
0.72
0.61
0.46
0.46
0.35
0.27
0.8
0.6
0.4
0.2
0.0
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Brain
1.92
1.20
1.14
0.83
0.60
0.58
2.0
1.5
1.0
0.5
0.0
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Gills
2.0
1.5
1.0
0.5
0.0
0.72
0.71
1.76
0.84
1.15
1.97
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

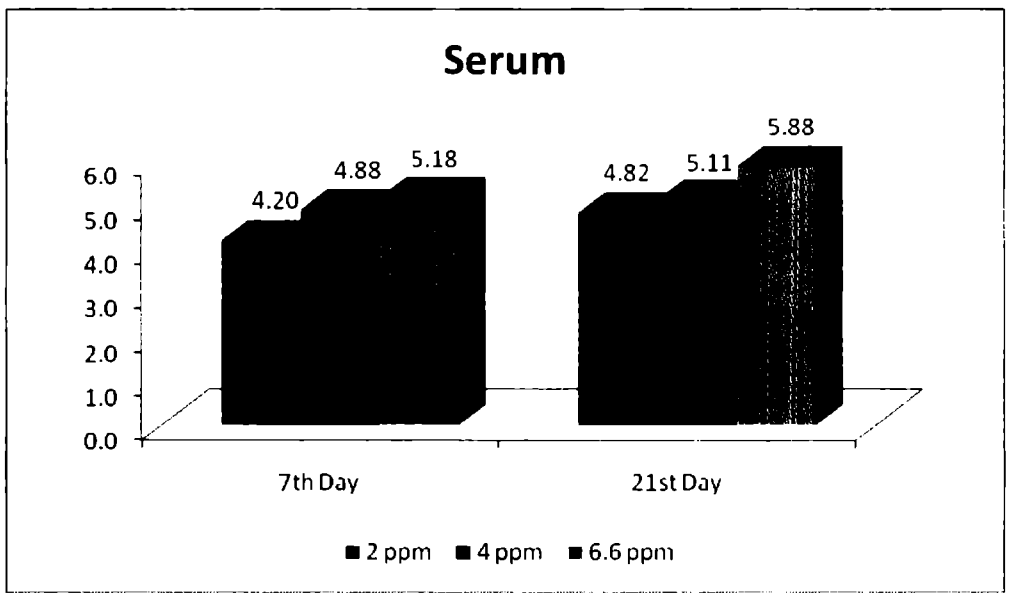
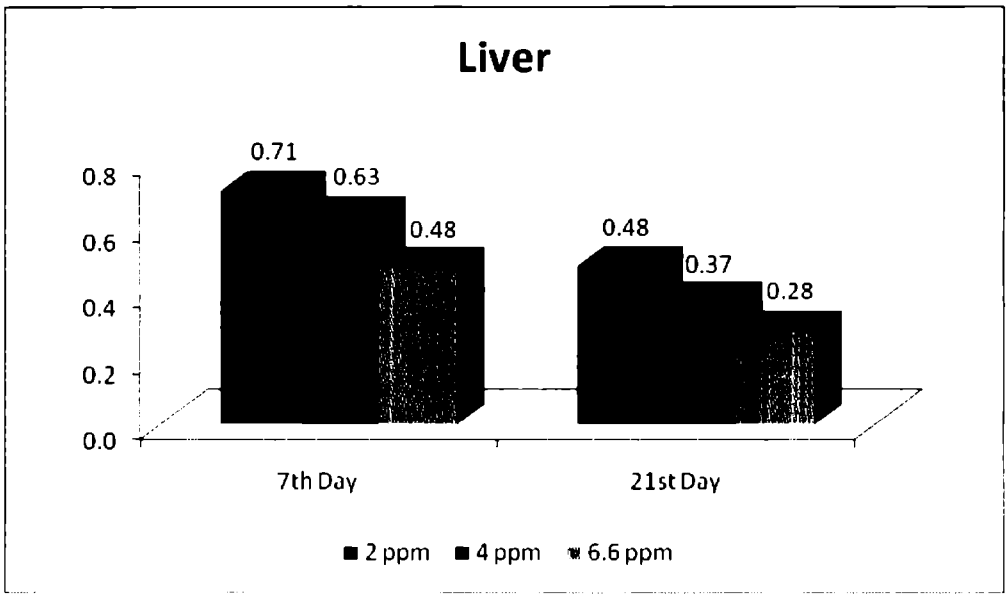
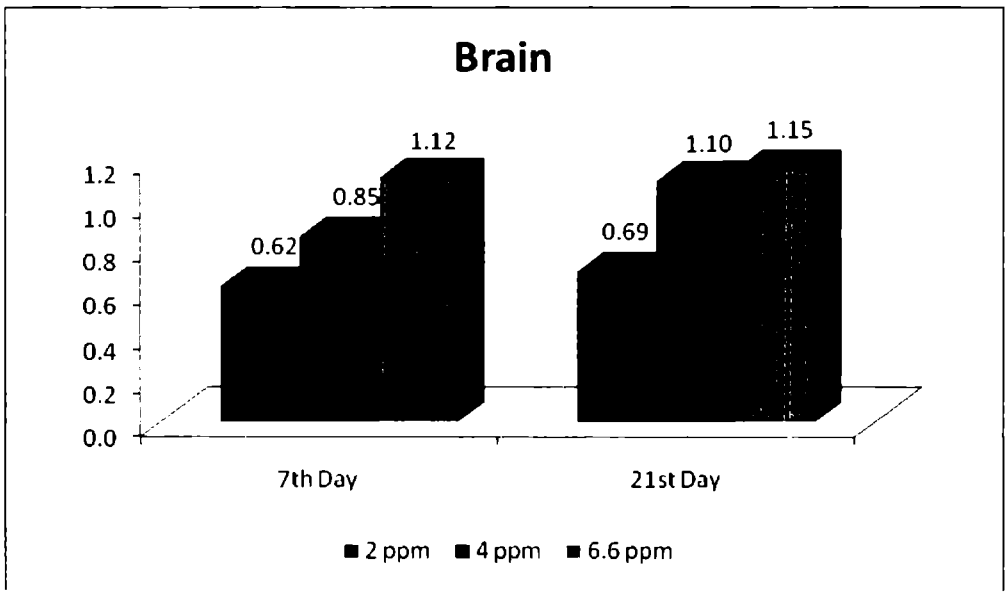

Serum
6.0
5.0
4.0
3.0
2.0
1.0
0.0
4.20
4.88
5.18
4.82
5.11
5.88
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

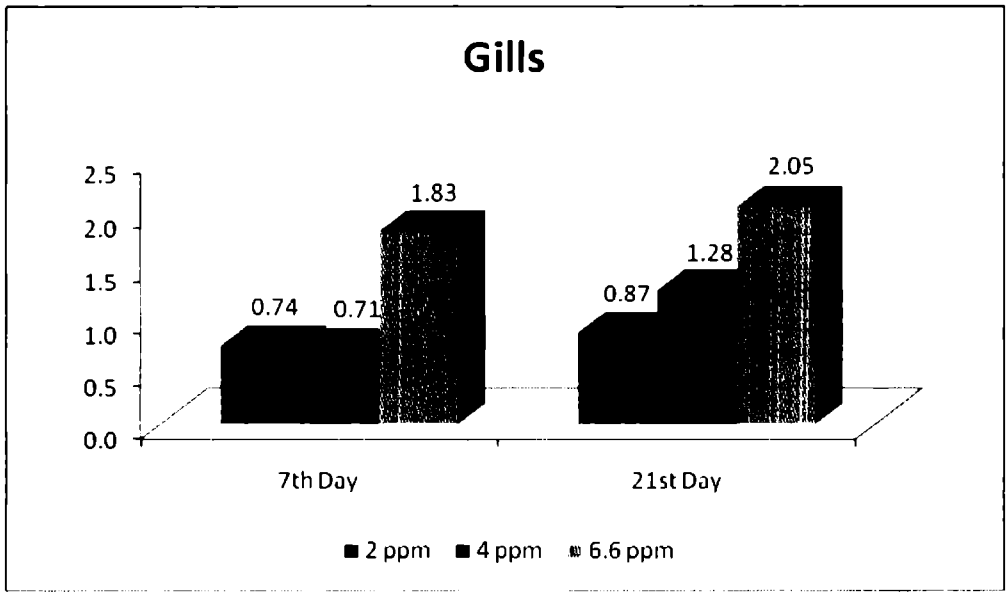
Table 6.6

Effect of cadmium on the acid phosphatase activity on the tissues of *Etroplus maculatus*

Tissue and serum	Control	0.33ppm		0.67ppm		1.2ppm	
		7^{th}	21^{st}	7^{th}	21^{st}	7^{th}	21^{st}
Liver	0.9899	0.7081	0.4792	0.6319	0.3717	0.4781	0.2834
	0.9798	0.7063	0.4791	0.6328	0.3719	0.4789	0.2838
	0.9789	0.7061	0.4793	0.6327	0.3809	0.4783	0.2837
	0.9786	0.7064	0.4795	0.6329	0.3728	0.4781	0.2832
	0.9793	0.7093	0.4796	0.6318	0.3712	0.47790	0.2828
	0.9893	0.7091	0.4798	0.6323	0.3715	0.47850	0.2835
Brain	0.5884	0.6194	0.6871	0.8463	1.1008	1.1146	1.1546
	0.5881	0.6193	0.6879	0.8468	1.1006	1.1158	1.1523
	0.5885	0.6199	0.6873	0.8469	1.1003	1.1163	1.1515
	0.5889	0.6189	0.6864	0.8464	1.1009	1.1125	1.1529
	0.5891	0.6199	0.6869	0.8459	1.1002	1.1159	1.1548
	0.5889	0.6189	0.6865	0.8468	1.1005	1.1149	1.1536
Gill	0.6307	0.7345	0.8572	0.7118	1.1712	1.8181	2.0494
	0.6219	0.7443	0.8672	0.7112	1.1719	1.8175	2.0521
	0.6394	0.7335	0.8671	0.7116	1.1710	1.8783	2.0501
	0.6308	0.7453	0.8669	0.7115	1.1814	1.8180	2.0513
	0.6398	0.7389	0.8678	0.7112	1.1729	1.8189	2.0524
	0.6318	0.7442	0.8670	0.7110	1.7918	1.8178	2.0520
Serum	4.0124	4.203	4.9118	4.9063	5.1278	5.1882	5.9261
	4.0284	4.201	4.919	4.9065	5.1293	5.1884	5.9564
	4.0129	4.213	4.9118	4.9010	5.1284	5.1889	5.9831
	4.0253	4.215	4.9189	4.9025	5.1285	5.1880	5.9842
	4.0119	4.208	4.9181	4.9010	5.1299	5.1879	5.999
	4.0254	4.208	4.918	4.9089	5.1289	5.1869	5.9841


Liver
0.71
0.63
0.48
0.48
0.37
0.28
0.8
0.6
0.4
0.2
0.0
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Brain
1.12
0.85
0.62
1.10
1.15
0.69
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm


Gills
2.5
2.0
1.5
1.0
0.5
0.0
0.74
0.71
1.83
0.87
1.28
2.05
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

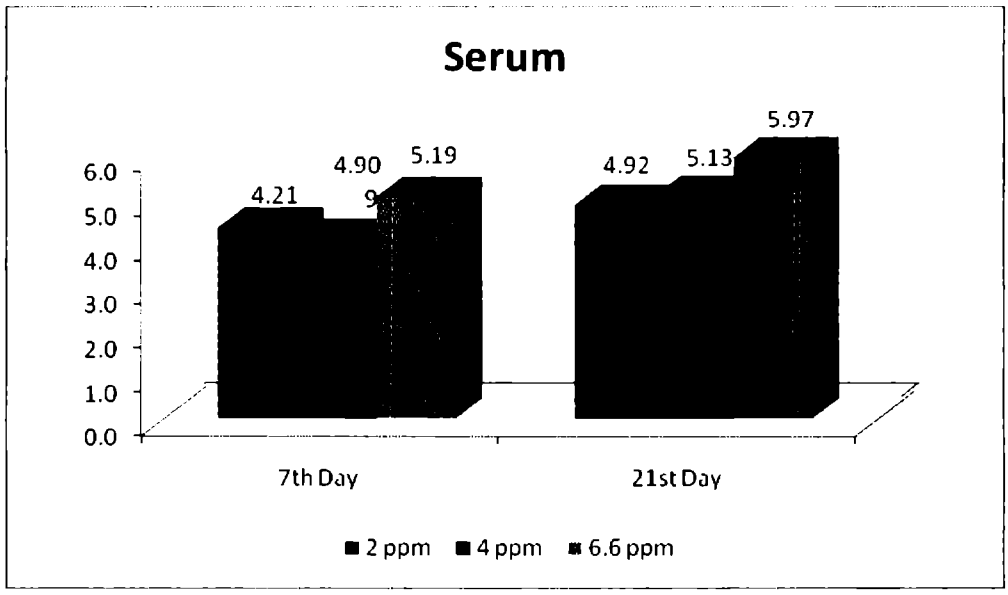

Serum
6.0
5.0
4.0
3.0
2.0
1.0
0.0
4.21
4.90
5.19
4.92
5.13
5.97
7th Day
21st Day
2 ppm
4 ppm
6.6 ppm

Table 6.7

Descriptive Statistics for Alkaline Phosphatase activity of lead exposure

Concentration	Tissue	Mean	Minimum	Maximum	Range	Std. Deviation
Control	Liver	1.078333	1.0700	1.0810	.0110	.0041312
	Brain	.917333	.9160	.9190	.0030	.0012111
	Gill	.741667	.7200	.7600	.0400	.0147196
	Serum	4.577833	4.5600	4.5960	.0360	.0141622
	Total	1.828792	.7200	4.5960	3.8760	1.6258813
2 ppm	Liver	.943333	.8500	1.0380	.1880	.0887052
	Brain	.550917	.0360	1.0190	.9830	.4912973
	Gill	.859167	.8100	.8900	.0800	.0231432
	Serum	5.374842	5.0181	5.6980	.6799	.3090228
	Total	1.932065	.0360	5.6980	5.6620	2.0341018
4 ppm	Liver	.886833	.8710	.8980	.0270	.0099620
	Brain	1.670833	1.3010	2.0300	.7290	.3632732
	Gill	1.744167	1.5200	1.9800	.4600	.2144531
	Serum	5.351667	4.7190	5.9400	1.2210	.5616065
	Total	2.413375	.8710	5.9400	5.0690	1.7804206
6.6 ppm	Liver	.718250	.7000	.7410	.0410	.0152323
	Brain	2.417167	2.0700	2.7900	.7200	.3541440
	Gill	2.625000	2.3300	2.9700	.6400	.2924038
	Serum	6.420667	6.0000	6.8610	.8610	.4264288
	Total	3.045271	.7000	6.8610	6.1610	2.1282122

Table 6.8

Descriptive Statistics for Enzymes on lead exposure

Experiment	Mean	Minimum	Maximum	Range	Std. Deviation
Alkaline Phosphatase	2.37	0.04	6.86	6.82	1.9810
Acetyl Cholinesterase	0.09	0.00	0.37	0.37	0.0803
Acid Phosphate	1.88	0.28	6.00	5.72	1.8206
Total	1.45	0.00	6.86	6.86	1.8341

Table 6.9

Analysis of Variance of Enzymes activity on exposure to lead

Source	Sum of Squares	df	Mean Square	F	P-value
Experiment	482.176	2	241.088	99.826	0.000
Error	1209.958	501	2.415		
Total	1692.134	503			

Table 6.10

Analysis of Variance for Alkaline Phosphatase activity on exposure to lead

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	29.923	2	14.961	78.055	0.000
Day	7.950	1	7.950	41.478	0.000
Tissue	578.509	3	192.836	1006.039	0.000
Error	30.669	160	0.192		
Total	655.340	167			

Table 6.11

Descriptive Statistics for Acid Phosphate activity on exposure to lead

Concentration	Tissue	Mean	Minimum	Maximum	Range	Std. Deviation
Control	Liver	.982633	.9786	.9899	.0113	.0054147
	Brain	.588650	.5881	.5891	.0010	.0003782
	Gill	.632400	.6219	.6398	.0179	.0066305
	Serum	4.019383	4.0119	4.0284	.0165	.0077370
	Total	1.555767	.5881	4.0284	3.4403	1.4613205
2 ppm	Liver	.593483	.4791	.7093	.2302	.1191430
	Brain	.653200	.6189	.6879	.0690	.0353236
	Gill	.802825	.7335	.8678	.1343	.0656504
	Serum	4.562133	4.2010	4.9190	.7180	.3699058
	Total	1.652910	.4791	4.9190	4.4399	1.7099134
4 ppm	Liver	.502867	.3712	.6329	.2617	.1353171
	Brain	.973533	.8459	1.1009	.2550	.1326650
	Gill	.994042	.7110	1.7918	1.0808	.3407505
	Serum	5.016583	4.9010	5.1299	.2289	.1172289
	Total	1.871756	.3712	5.1299	4.7587	1.8560723
6.6 ppm	Liver	.380850	.2828	.4789	.1961	.1017838
	Brain	1.134142	1.1125	1.1548	.0423	.0200337
	Gill	1.939658	1.8175	2.0524	.2349	.1176959
	Serum	5.580100	5.1869	5.9990	.8121	.4098712
	Total	2.258688	.2828	5.9990	5.7162	2.0275336

Table 6.12
Analysis of Variance for Acid Phosphate activity on exposure to lead

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	9.033	2	4.517	39.005	0.000
Day	1.332	1	1.332	11.506	0.001
Tissue	521.802	3	173.934	1502.079	0.000
Error	18.527	160	0.116		
Total	553.542	167			

Table 6.13
Descriptive Statistics for Acetyl Cholinesterase activity on exposure to lead

Concentration	Tissue	Mean	Minimum	Maximum	Range	Std. Deviation
Control	Liver	.105033	.1023	.1094	.0071	.0031576
	Brain	.362200	.3538	.3727	.0189	.0061491
	Gill	.196150	.1924	.1989	.0065	.0026129
	Serum	.262650	.2623	.2629	.0006	.0002429
	Total	.231508	.1023	.3727	.2704	.0960188
2 ppm	Liver	.049742	.0312	.0783	.0471	.0155041
	Brain	.104608	.0921	.1124	.0203	.0070901
	Gill	.090675	.0801	.1008	.0207	.0103574
	Serum	.164967	.1271	.2019	.0748	.0383914
	Total	.102498	.0312	.2019	.1707	.0467104
4 ppm	Liver	.045375	.0210	.2252	.2042	.0567401
	Brain	.082067	.0713	.0924	.0211	.0098302
	Gill	.036342	.0286	.0458	.0172	.0076940
	Serum	.111942	.0761	.1471	.0710	.0362939
	Total	.068931	.0210	.2252	.2042	.0450273
6.6 ppm	Liver	.022708	.0040	.1240	.1200	.0322410
	Brain	.055792	.0418	.0694	.0276	.0118179
	Gill	.018517	.0142	.0228	.0086	.0037316
	Serum	.073633	.0388	.1087	.0699	.0360526
	Total	.042663	.0040	.1240	.1200	.0335091

Table 6.14

Analysis of Variance for Acetyl Cholinesterase activity on exposure to lead

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	0.086	2	0.043	31.368	0.000
Day	0.020	1	0.020	14.787	0.000
Tissue	0.222	3	0.074	53.780	0.000
Error	0.220	160	0.001		
Total	1.077	167			

Table 6.15

Descriptive Statistics of enzymes on exposure of Cadmium and lead

		Mean	Minimum	Maximum	Range	Std. Deviation
Al P	**Lead**	2.45	0.71	8.88	8.17	2.0159
	Cadmium	2.37	0.04	6.86	6.82	1.9810
	Total	2.41	0.04	8.88	8.84	1.9959
Ac C	**Lead**	0.10	0.01	1.29	1.28	0.1301
	Cadmium	0.09	0.00	0.37	0.37	0.0803
	Total	0.10	0.01	1.29	1.28	0.1080
Ac P	**Lead**	1.88	0.08	6.08	6.00	1.8400
	Cadmium	1.88	0.28	6.00	5.72	1.8206
	Total	1.88	0.08	6.08	6.00	1.8276

Table 6.16

Independent Sample t-test

	t-value	df	p-value
Al P	0.342	334	0.733
Ac C	0.543	334	0.587
Ac P	0.014	334	0.989

Table 6.17

Descriptive Statistics of Enzyme analysis on exposure to lead

Experiment	Mean	Minimum	Maximum	Range	Std. Deviation
Alkaline Phosphatase	2.45	0.71	8.88	8.17	2.0159
Acetyl Cholinesterase	0.10	0.01	1.29	1.28	0.1301
Acid Phosphate	1.88	0.08	6.08	6.00	1.8400
Total	1.48	0.01	8.88	8.87	1.8654

Table 6.18

Analysis of Variance of Enzyme analysis on exposure to lead

Source	Sum of Squares	df	Mean Square	F	P-value
Experiment	503.382	2	251.691	101.128	0.000
Error	1246.911	501	2.489		
Total	1750.294	503			

Table 6.19

Descriptive Statistics for Alkaline Phosphatase on exposure to Cadmium

Concentration	Tissue	Mean	Minimum	Maximum	Range	Std. Deviation
Control	Liver	1.078667	1.0750	1.0810	.0060	.0025820
	Brain	.900000	.8900	.9100	.0200	.0089443
	Gill	.751667	.7500	.7600	.0100	.0040825
	Serum	4.568500	4.5600	4.5790	.0190	.0075033
	Total	1.824708	.7500	4.5790	3.8290	1.6225268
2 ppm	Liver	.921417	.7900	1.0180	.2280	.0983024
	Brain	1.030333	1.0100	1.0900	.0800	.0217813
	Gill	.856667	.8300	.8900	.0600	.0192275
	Serum	5.384750	5.0700	5.7200	.6500	.3130211
	Total	2.048292	.7900	5.7200	4.9300	1.9542072
4 ppm	Liver	1.540750	.8500	8.8800	8.0300	2.3113037
	Brain	1.708333	1.2890	2.0980	.8090	.3904683
	Gill	1.593333	1.5500	1.9400	.3900	.1094061
	Serum	5.353333	4.7100	5.9400	1.2300	.5858845
	Total	2.548937	.8500	8.8800	8.0300	2.0125061
6.6 ppm	Liver	.735333	.7100	.7990	.0890	.0248901
	Brain	2.505750	2.1400	2.8810	.7410	.3172791
	Gill	2.566667	2.2300	2.9000	.6700	.3264780
	Serum	6.417583	5.0800	6.9700	1.8900	.6044156
	Total	3.056333	.7100	6.9700	6.2600	2.1290324

Table 6.20

Analysis of Variance for Alkaline Phosphates on exposure to Cadmium

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	24.388	2	12.194	22.586	0.000
Day	8.897	1	8.897	16.479	0.000
Tissue	548.160	3	182.720	338.443	0.000
Error	86.381	160	0.540		
Total	678.683	167			

Table 6.21

Descriptive Statistics for Acetyl Cholinesterase activity on exposure to Cadmium

Concentration	Tissue	Mean	Minimum	Maximum	Range	Std. Deviation
Control	**Liver**	.100283	.1002	.1004	.0002	.0000753
	Brain	.353233	.3529	.3537	.0008	.0003445
	Gill	.197583	.1971	.1979	.0008	.0003764
	Serum	.252367	.2521	.2528	.0007	.0002582
	Total	.225867	.1002	.3537	.2535	.0934776
0.33 ppm	**Liver**	.034550	.0271	.0405	.0134	.0060185
	Brain	.098067	.0827	.1099	.0272	.0118268
	Gill	.085583	.0691	.1007	.0316	.0156610
	Serum	.261175	.1271	1.2860	1.1589	.3248237
	Total	.119844	.0271	1.2860	1.2589	.1793618
0.67 ppm	**Liver**	.018175	.0102	.0270	.0168	.0077271
	Brain	.073017	.0614	.0829	.0215	.0091032
	Gill	.031900	.0252	.0462	.0210	.0064028
	Serum	.159942	.0681	.6931	.6250	.1724440
	Total	.070758	.0102	.6931	.6829	.1006289
1.2 ppm	**Liver**	.060483	.0093	.1290	.1197	.0591676
	Brain	.044333	.0329	.0549	.0220	.0089688
	Gill	.019542	.0131	.0340	.0209	.0066179
	Serum	.070592	.0291	.1097	.0806	.0374975
	Total	.048737	.0093	.1290	.1197	.0394619

Table 6.21

Analysis of Variance for Acetyl Cholinesterase activity on exposure to Cadmium

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	0.127	2	0.064	5.530	0.005
Day	0.006	1	0.006	0.494	0.483
Tissue	0.416	3	0.139	12.062	0.000
Error	1.840	160	0.012		
Total	2.828	167			

Table 6.22

Descriptive Statistics for Acid Phosphate activity on exposure to Cadmium

Concentration	Tissue	Mean	Minimum	Maximum	Range	Std. Deviation
Control	Liver	.982833	.9760	.9870	.0110	.0040702
	Brain	.587583	.5854	.5884	.0030	.0011720
	Gill	.630617	.6294	.6314	.0020	.0008305
	Serum	4.011833	4.0110	4.0120	.0010	.0004082
	Total	1.553217	.5854	4.0120	3.4266	1.4584495
0.33 ppm	Liver	.591867	.4601	.7890	.3289	.1377575
	Brain	.588158	.0769	.6810	.6041	.1651662
	Gill	.778508	.7145	.8395	.1250	.0602517
	Serum	4.509417	4.2000	4.8190	.6190	.3209170
	Total	1.616987	.0769	4.8190	4.7421	1.6999643
0.67 ppm	Liver	.481758	.3489	.6210	.2721	.1354711
	Brain	1.371892	.8191	6.0790	5.2599	1.4879325
	Gill	.928742	.7008	1.1542	.4534	.2296647
	Serum	4.998183	4.8710	5.1181	.2471	.1195187
	Total	1.945144	.3489	6.0790	5.7301	1.9525404
1.2 ppm	Liver	.364467	.2693	.4670	.1977	.0952289
	Brain	1.166792	1.1189	1.4150	.2961	.0809987
	Gill	1.866833	1.7190	1.9910	.2720	.1113021
	Serum	5.530333	5.1690	5.9800	.8110	.3702256
	Total	2.232106	.2693	5.9800	5.7107	2.0076042

Table 6.23

Analysis of Variance for Acid Phosphate

Source	Sum of Squares	df	Mean Square	F	P-value
Concentration	9.094	2	4.547	16.960	0.000
Day	1.871	1	1.871	6.977	0.009
Tissue	508.594	3	169.531	632.308	0.000
Error	42.898	160	0.268		
Total	565.400	167			

6.4 DISCUSSION

Blood glucose is a sensitive reliable indicator of environmental stress in fish. From the results, it is clear that Cd as shown by the elevated blood glucose level affected as astress or on fish. Cd induced hyperglycemia with decreased in liver glycogen in catfish, Heteropneustes fossilis (Sastry and Subhadra, 1985). Soengas et.al (1996) suggested that hyperglycemia occurred in Atlantic salmon (Salmo salar) after toxicity with cadmium, may be due to changes in liver carbohydrate metabolism (activation of liver glycogenolysis and glycolysis) as well as increased level of plasma glucose and lactate.

Sastry and Shukla (2007) studied the haematological, biochemical and enzymological alterations produced on exposure of channa punctatus to sublethal concentration (1.12 mg/l). Fish were hypoglycemic and hypolactemic. The pyruvate content of blood and liver decreases in acute and all atages of chronic exposure except for 30 days where significant increase was recorded.Depletion was noted in the total protein and glycogen content of liver and muscle.

Total protein is a frequently parameter of metal poisoning in fish. However, data available did not allow to assessment of the direction of these changes, since the same metal may cause both an increase and a decrease on total protein. There were no changes in plasma total protein in fish exposed to cadmium at 15 days. While these values were decreased significantlt in fish exposed to Cd . Adel (2007) showed that liver protein and muscle total protein significantly decreased in fish exposed to Cd. This result may be attributed to the great demands and cellular

damage that occurred in the tissues of Cd- toxicated fish and Cd topxicity may be possible cause protein brealdown.

Lin et.al., (2001) noticed deposition of the particulate lead on the gill surface temporarily that sloughed along with the slime into the water flow. The slime secreted by *Etroplus maculates* especially after lead nitrate exposure contains a mixture of neutral and acidic glycoproteins. This due to masking of the heavy metal salts with the mucus from the respiratory surfaces of *Etroplus maculates*, the toxicity of irritant substantially reduces.

Chandravathy et.al., (1987) found an increase in the total protein concentration of muscle, liver, gills, kidney and brain of the climbing perch *A.scandens* following lead nitrate exposure. Elevation in the ratio of protein content was also noticed after treatments with other heavy metals (e.g. mercury, Radi and Matkovics, 1988 and cadmium, Rajanna et.al., 1981 and Joshi and Bose, 2002.). Increased protein synthesis against the toxicity of heavy metals leads to the adaptation of organisms to a toxic environment and also induces tolerant stress (Ferragut et.al., 1991). Continuous exposure of fresh water fish, *H. fossilis* to sub lethal concentration of lead nitrate for 35 days however causes decreased soluble proteins and RNA contents in its liver (Jain et.al., 1996). While comparing the effects of lead chloride, lead acetate and aluminum compounds on the air breathing cat fish *Clarias lazera*, Abdel hamid and el-Ayouty (1991) noticed that lead chloride decreased protein contents of kidney. Xie. et.al. (1986) reported a reduction in serum protein contents of bighead carp (Aristichthys nobilis) following exposure to lead. Following lead nitrate exposure, the total lipid contents of *Etroplus maculates* decrease in the early stages of exposure. This may perhaps be due to their use in extensive slime secretion. Subsequently at various stages, the volume of the lipids fluctuated. The lipid content also becomes abnormal at several stages. Following cadmium exposure, Fantin et.al., (1992) however found increased lipid contents. Similarly the total lipid contents in the liver, muscle, kidney and ovary of *Clarias batrachus* increased following exposure to carbofuran toxicity (Begum and Vijayaraghavan, 2001).

Total lipids in plasma increased significantly in fish exposed to cadmium (Shalaby, 2007). Shalaby (2001) reported that the absorption of excess heavy metal disturbed the metabolism of lipid.

The activity of AST and ALT enzymes in blood may also be used as a stress indicator. The significant changes in activities of these enzymes in blood plasma indicate tissue impairment caused by stress (James et.al., 1991 and Svoboda 2001). In the present study, there were significant changes in AST and ALT activities in plasma of fish exposed to cadmium compared to the control group. The increase concentration of AST and AL on blood plasma indicates impairement of parnchymatous organs (namely liver). In addition to the increase of plasma AST and ALT may be attributed to the hepatocellular damage or cellular degradation by these heavy metal, perhaps in liver, heart or muscle (Yamawaki et.al., 1986). These results are in agreement with those of Shalaby (1997) who found that sublethal concentration of Cd caused significant increase in AST and ALT of common carp after 7 and 15 days.

The blood glucose level (60 .32 mg/ml) in nile tilapia was similar to those reported by Tavares –Dias et.al (1999) for "pacu" (76.4 mg/ml). However, this value is significantly higher than the value obtained for channel caty fish Icta lurus meals (39.7 mg/ml). Seasonal temperature changes may affect blood sugar levels. . The seasonal difference in blood glucose lvel may also be due to the different sugar metabolism in the various seasons. A pointed by Chavin and Young (1970). temperature affects the blood sugar levels, but the pattern is inconsistent.

The decrease of ALP activity in plasma due to Cd toxicity was similar to that obtained by Shalaby (2007) who ecorded a significant reduction in plasma ALP activity in Oreochromis niloticus exposed to sublethal concentration of Cd.. Sastry and Subhadra (1985) also recorded that a significant reduction in ALP in liver and kidney of cat fish Hetropneustes fossilis after toxication with Cd. This decrease may be due to the damage and dysfunction of the liver. The ACP activity increased significantly in fish esposed to cadmium more than the control. This result is in agreement with Shalaby (2007) .Sasty and Subhadra (1985) who found that a

significant increased in ACP in Kidney of cat fish, Hetropneustes fossilis after toxication with Cd.

Depletion of the glycogen in the hepatocytes is usually is uaually found in stressed animals (Hinton & Lauren, 1990; Wilhelm Filho et.al., 2001), because the glycogen acts as a reserve of glucose to supply the higher energetic demand occurring in such situations (Panepucci et.al., 2001).However, the tendency of the animals to show less glycogen in the summer may be related to the higher metabolic rate caused by the higher temperatures.

AchE showed mo changes after Cd exposure. Both livers GOT and GPT activities were increased by the metal by 63 and 98 %. The Cd exposure resulted in an irreversible alteration in GOT activity Result indicated that the sublethal Cd concentrations are stressful to carp particularly with reference to branchial enzymes which may disrupt the osmotic and ionic balance of the animals (De la et.al., 2000).

De la Torre et.al., (2002) pointed out that Ach E activities can be partially attributed to long-lasting raised concentration of dissolved heavy metal..

Since the acute sampling procedure (Gallaugher and Farrell, 1998) does not totally exclude stress effects (Anderson et.al., 1985: Bollard et.al., 1993: Roche & Boge, 1996; Barton et.al., 1998), it can be argued that the physical stress of manipulation may have affected haematological parameters. It appears that the general response to stress is a rapid increase in catecholamines followed by cortisol and glucose release, and changes in several haematological parameters, which in some cases involve Hct and Hb (Gallauger and Farrell, 1998; Nikinmaa and Salama, 1998; Kieffer, 2000). Probably red blood cells experience a swelling effect induced by catecholamine, cortisol and glucose release, coupled with release of erythrocytes from the spleen (Gallaugher and Farrell, 1998; Cooper et.al., 2001)

6.5 CONCLUSION

From the above discussion, it is clear that heavy metal toxicity seriously impairs various metabolic functions of the fish *Etroplus maculates*, reflected as alterations in various biochemical constituents. AAT, ALAT ACP and ALP activity in plasma which are conventional indicators of liver injury are observed to be

increased in the heavy metal xposed *Etroplus maculates*. Plasma activity concentrations of AAT and ALAT are the most commonly used biochemical markers of hepatocellular necrosis. The increase of biomarker enzymes in plasma might be due to the necrosis of liver. The result revealed that heavy metals cadmium and lead affects the intermediary metabolism of *Etroplus maculates* and the assayed enzymes can work as good biomarkers of heavy metal contamination. The present data confirm that AChE to be a sensitive enzyme marker. Acetylcholinesterases, due to its rate of hydrolysis towards the substance acetylcholinesterase, due to its high rate of hydrolysis towards the substrate acetylcholine chloride, confirm its presence in *Etroplus maculates*. It is highly sensitive and is recommended as a useful biomarker in biomonitoring studies.

Chapter 7

Histopathological effects of heavy metals

HISTOPATHOLOGICAL EFFECTS OF HEAVY METALS

7.1 INTRODUCTION

Sub lethal levels of pollutants usually cause biochemical and physiological effects at the sub cellular level in an organism. There are various methods adopted to understand and explain the toxic effects in marine organisms. Initially, larger animals were screened for visible disease signs and abnormalities on skin and liver. In addition to that, tissue residue concentrations of toxicants and change in other metabolic biomarkers, but as more in depth information was required such as further characterization or screening for early pre-neoplastic changes, histopathology was introduced.

Histopathology is an essential tool in fish pathology, physiology and aquatic toxicology in fishes. Histopathological examination is widely recognized as a reliable method for disease diagnosis (Ferguson, 1989) and for assessing acute and chronic effects of exposure to toxicants at the cellular level in both marine and fresh water species (Hinton *et al.*, 1992). Histopathological techniques are rapid, sensible, reliable and comparatively inexpensive tools for the assessment of stress response to xenobiotics. Cytological and histopathological alterations provide a direct record of stress effects. Cell damage is a result of persistent or irreversible biochemical and sub cellular dysfunction caused by stress. Often stressed cells undergo irreversible structural and biochemical changes, which result in the alterations in the physiology of the animal. Assessment of histopathological manifestation provides insight into the degree of stress, susceptibility and adaptive capability of the stressed organism.

Histopathology has been used for many years to study the cellular basis of infectious and noninfectious diseases. Fish respond to various insults in many ways very similar to mammals. Therefore, fish histopathology utilizes knowledge gained over many years from human and veterinary pathology (Grizzle and Rogers, 1976;

Ferguson, 1989). Acute changes are seen when pollutant levels are high, while chronic duration is necessary to determine sub lethal aspects of change (Vander Oost *et al.*, 2003). Many of histological changes persist even after the toxicant exposure has ceased.

The aim of present study was to assess the degree of histopathological alterations in the liver, gills and kidney on exposure to sub lethal concentrations of lead and cadmium. Liver being the major organ involved in xenobiotic metabolism and excretion was considered ideal for the study. The study focused on gills because gills are the main respiratory organs in fishes and target for many aquatic pollutants in general; they are one of the most seriously affected organs because they have direct contact with the aquatic environment (Mishra *et al.*, 1985). The gills have widely been used as bio-indicator not only to detect lead and cadmium toxicity (Parashar and Banerjee, 2002), but also for analyses of several other pollutants (Nath *et al.*, 1989; Munshi and Singh 1992; Chandra and Banerjee, 2004). Kidney also selected for the study being the vital organ for detoxification and removal of toxic substances circulating in the blood stream (Klavercamp *et al.*, 1984 and Kent, 1998).

7.2 MATERIALS AND METHODS

Collection, transportation, acclimation and bioassay of/on *Etroplus maculatus* was performed, as described in the chapter 1 and 3.

The fishes of sizes ranges from 5-8 cm length and 4-8 g weight were selected for the study. Eight groups of ten acclimatized fishes were exposed to sub lethal concentrations of 2 different ppm of cadmium chloride and lead nitrate for a periods of 14 and 28 days. Parallel control groups of fishes were kept in 10 L of plain water, toxicant free. Feeding was allowed to both experimental and control fishes throughout the tenure of experiments. Entire experiment was repeated twice.

The fishes for experiments were divided into two main groups. The fishes were exposed to two different sub lethal concentrations of heavy metals cadmium and lead. The fishes were designated as FC and FL that are fish exposed to

cadmium and fish exposed to lead. In this FC further subdivided into FCa and FCb and this designated as the experimental group that receive two different sub lethal concentrations (ppm) of cadmium. This above groups divided in to FCa1 and FCa2. These are designated as fishes exposed to three different concentrations of cadmium for the period of 14 and 28 days The fishes exposed to Lead, FL subdivided into FLa and FLb and this are designated as the experimental group that receive two different sub lethal concentrations (ppm) of lead. The above groups again divided into FLa1 and FLa2 and these are designated as fishes exposed to two different concentrations of lead for 14 and 28 days.

Sublethal Cadmium dose (ppm)	**0.33 ppm**		**1.2 ppm**	
Group	FCa		FCb	
Subgroup	FCa1	FCa2	FCb1	FCb2
Period in days	14	28	14	28

Sublethal lead dose (ppm)	**2 ppm**		**6.6ppm**	
Group	FLa		FLb	
Subgroup	FLa1	FLa2	FLb1	FLb2
Period in days	14	28	14	28

Five fishes, each from the experimental as well as control groups were sacrificed on 14 th and 28 th day of exposure. Liver, gills and kidney tissues were dissected out and were washed to remove any debris. The organs that were removed were fixed in Bouin's fixative (Luna, 1992) for 24 hours and washed the tissues in running water for overnight. Then the tissues were treated with saturated solution of Lithium carbonate in 70 % alcohol to remove yellow color of picric acid. After softening, the tissues were stored in fresh 70 % alcohol. At this stage, the tissues can be stored for further processing. For gill tissue, after fixation in Bouin's fixative tissue should be placed in the decalcification solution to soften it. For that previously fixed tissue containing calcified components were washed in tap water for 8 hours, then rinsed in the distilled water and placed in decalcifying solution for

3 to 4 days and the solution changed daily for best results. After sufficient decalcification these were washed in tap water for 8 hours, rinsed in distilled water and stored in 70 % alcohol until processed.

Routine histological methods followed by Luna, 1968. Routine processing of tissue involved trimming into small pieces, dehydration with a series of alcohols followed by an organic solvent, and infiltration with paraffin. Blocks of paraffin containing the tissues were allowed to harden and then were cut into slices 3-6 mm wide. The slices were dried, deparaffinized, and stained with haematoxylin and eosin (H and E), Humason, (1979) and observed the histopathological effects under light microscopy, using a camera–mounted microscope to document findings. This procedure is as follows.

7.2.1 Dehydration

Dehydration of tissues was done with progressive higher grades of alcohol series as (70 %, 80 %, 90% and 95 % alcohol for 1 h, and two changes of absolute alcohol (100 %) alcohol for 1hour.

7.2.2 Clearing

For clearing, tissues were changed to alcohol –acetone solution (1: 1) for 30 min, and then to acetone (45 min – 1 hour), followed by acetone – xylene (1: 1) for 30 min, and to xylene alone (45 min – 1 hour). Tissues were then transferred to xylene: wax (30 min).

7.2.3 Infiltration and Embedding

Tissues were in filtered in 2-3 changes of molten paraffin of melting point 58-62 0C, and then embedded in wax at 58-60 0 C, made into blocks which were labeled, and stored in polythene cover.

7.2.4 Sectioning

Paraffin blocks were trimmed to suitable size and sections of tissue were cut using a microtome at 5-7 µm thickness. The resulting ribbons containing tissue sections were fixed on to glass slides using Mayer's egg albumin glycerol (1:1 v/v) as an adhesive.

7.2.5 Staining technique followed with Ehrlich's haematoxylin-Eosin (H/E) stains (Pearse, 1985)

Rinsed the slides twice in xylene: 5 min each

Transferred slides to xylene: absolute alcohol mixture (1:1): 1 min.

Slides passed sequentially through absolute alcohol, 80% alcohol and 50% alcohol: 1 min each.

Washed in running tap water: 5 min.

Stained in Haematoxylin: 2 min.

Washed in running tap water: 5 min.

Dipped once in acid: absolute alcohol mixture (0.5: 100).

Washed in running tap water: 5 min.

Stained in Eosin : 1 min.

Slides dehydrated sequentially through 50 %, 80% and absolute alcohol: 1 min.

Dipped once in running tap water.

Blotted slide on filter.

Rinsed in xylene: 5 min.

Rinsed in xylene : till clear.

Mounted in D.P.X and cover slips were put without locking any air bubbles. Sections were observed and photographed under light microscope (OLYMPUS BX 41TF, Japan.

Composition of fixative and stain used for the study

Bouin's Fixative was used as fixative. It is composed of the following ingradients.

Saturated aqueous solution of Picric acid	-	75 ml
Formalin concentrated (40 % aqueous solution of formaldehyde)	-	25 ml
Glacial acetic acid	-	5 ml

Fixed tissue should be transferred to 70 % alcohol.

Decalcification solution

Formic acid (85%)	-	1 part
Ethyl alcohol (95%)	-	1 part
10% sodium acetate in 1 % tricholro acetic acid	-	1 part

Ehrlich's Haematoxylin was used for staining. It is composed of following ingredients.

Distilled water	-	10 ml
100% alcohol	-	100 ml
Glycerin	-	100 ml
Glacial acetic acid	-	10 ml
Haematoxylin	-	2 g

Aluminum ammonium sulphate (or potassium alum) – 20 g

Eosin (0.5 %) as counter stain has the following composition

Eosin	-	500 mg
70 % alcohol	-	100 ml

7.3 RESULTS

During the duration of the experiment, the number of fish that died less than 5 % was insignificant relative to the total population, and the pattern of death could not be attributed to lead and cadmium exposure. Lesions were, however, observed in the liver, gills and kidney of sampled fish for all lead and cadmium treatments at all exposure concentrations and durations. The patterns of occurrence and degree of alterations were positively related with the concentration of lead and the duration of exposure. The control group showed normal without any alterations for all organs throughout the duration of the experiment.

Several changes were observed in the liver, gills and kidney tissues of animals exposed to different concentrations of lead and cadmium and different time periods. By comparing the histological structure of the liver, gills and kidney of the control and of metal exposed fishes, the following observations were made.

7.3.1 Histopathology of liver

7.3.1.1 Liver in control fish

The liver is composed of parenchyma cells. The liver parenchyma was divided into irregularly shaped lobules separated by hepatopancreas and associated connective tissue. Within the parenchyma, eight to ten hepatocytes were radially arranged around a central sinusoid in a tubular unit. Four to six more sinusoids also surround the periphery of the tubular unit a fairly regularly spaced intervals. Tubular units ran irregularly through the parenchyma. The sinusoids were lined with endothelial cells. Stellate reticuloendothelial cells (Kupffer cells) were not identified. The veins surrounded by liver or hepatic parenchyma or pancreatic tissue. The veins were sometimes accompanied by an artery or a bile duct. Hepatopancreas were polyhedral cells. Hepatopancreas consisted of a central portal vein surrounded by a basement membrane and an outer layer of connective tissue, both containing reticular fibers.

Liver of control fishes were devoid of any pathological changes and hepatocytes were seen as well arranged structures in histological sections.

7.3.1.2 Changes in liver observed on exposure to 0.33 ppm and 1.2 ppm of cadmium chloride for 14 th and 28 th day of exposure

At low concentrations of cadmium (0.33 ppm) at the end of 14 th day of exposure, there was an increased occurrence of stromal connective tissue. Still, the normal structure of the liver retained. The major histological changes identified in liver tissue including hyalinization, congestion of blood vessels, increased vacuolation associated with lipid accumulation and cellular swelling.

At the end of 28 th day of exposure necrosis of the cells were more severe. Nuclear pyknosis in the majority of hepatic cells were noticed.

At higher concentration of (1.2 ppm) of cadmium at the end of 14 th day fibrosis and more necrotic cells were observed. Nuclei were all pyknotic, swelling of hepatocytes, cytoplasmic generation and granulation of cytoplasm were all assessed. Total damage of the structure of the liver tissue was observed at the end of 28 day of exposure. Necrosis of the cells were also seen. Presence of large interportal spaces with hypertropic blood vessels and hemolized content were seen.

A rich leukocyte infiltrate was observed into the interlobular connective tissue. Liver tissue with more ceroid pigments was seen.

7.3.1.3 Changes in liver observed on exposure to 2 ppm and 6.6 ppm lead nitrate for 14 and 28 days

At lower concentration of lead nitrate on the 28 th day shows aggregates of round cells with large blue staining nuclei were clearly visible. Liver shows degenerative changes in the parenchyma. Cellular damage of tissues and tissues devoid of nuclei were seen in the liver. Vacuolar degeneration and cellular atrophy and hemolysis of red blood corpuscles appeared on the 14 th day of exposure.

At higher concentration of lead nitrate on the 14 th day of exposure the main alterations found in the liver cells were irregular shaped nuclei, nuclear hypertrophy, nuclear vacuolation and the presence of eosinophilic granules in the cytoplasm were noticed. On the 28 th day of exposure bile stagnation, cytoplasmic, nuclear degeneration, melanomacrophages and necrosis of the tissue were more noticed.

Histopathological alterations in the liver of *Etroplus maculatus* on exposure to sublethal concentrations of cadmium chloride (Haemotoxylin & Eosin)

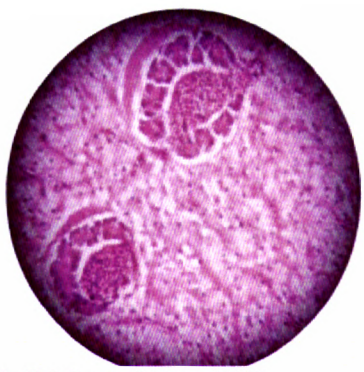


Fig 7.1 Control Liver (20X)

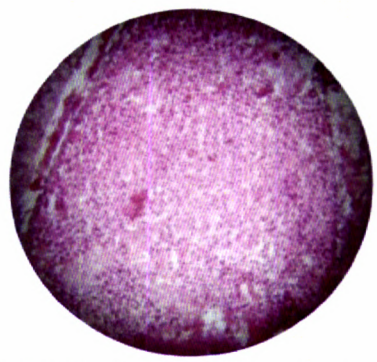


Fig 7.2 Blood clotting in Hepatocytes (20X)

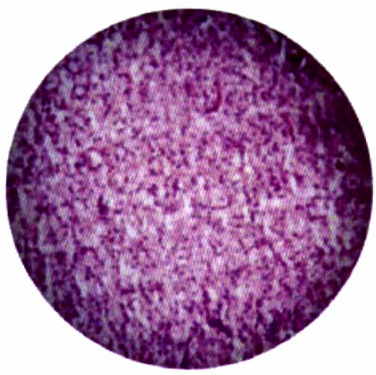


Fig 7.3 Development of Necrotic area (20X)

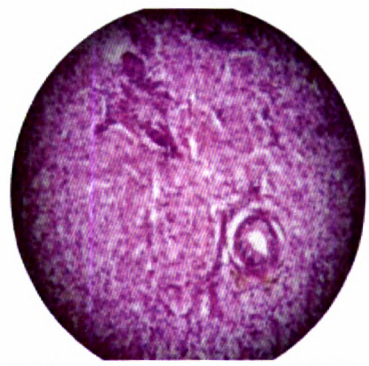


Fig 7.4 Leuckocyte infiltration (20X)

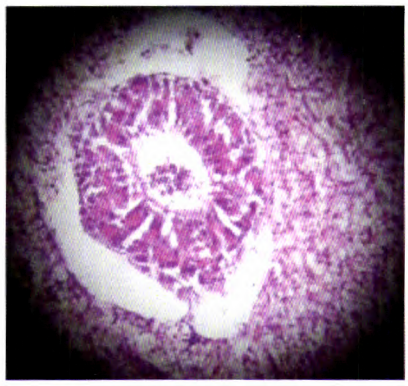


Fig 7.5 Rupture of Hepatocytes (40X)

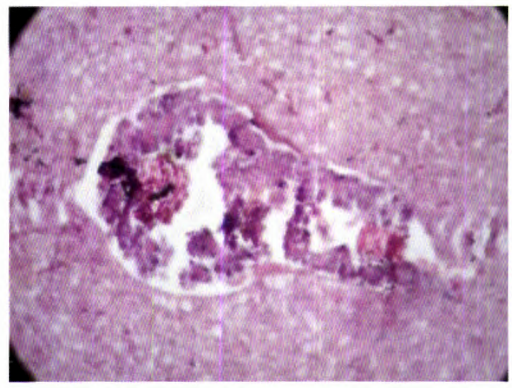


Fig 7.6 Hemorrhage in Hepatocytes(40X)

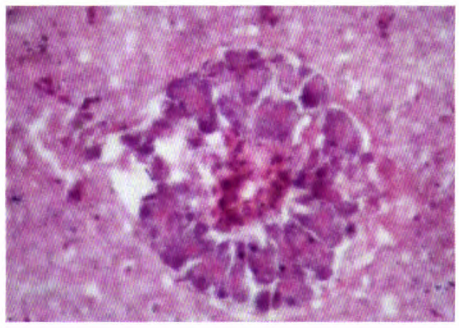


Fig 7.7 Leucocyte infiltration (40X)

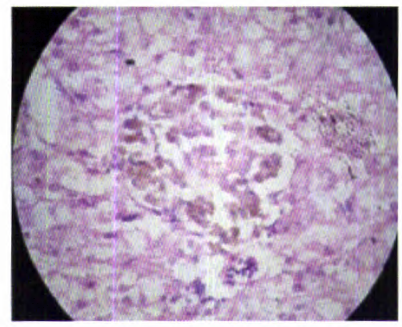


Fig 7.8 Mild deposit of Haemosiderin (40X)

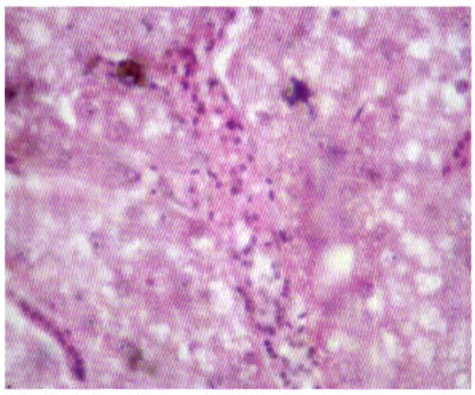


Fig 7.9 Vaculation (40X)

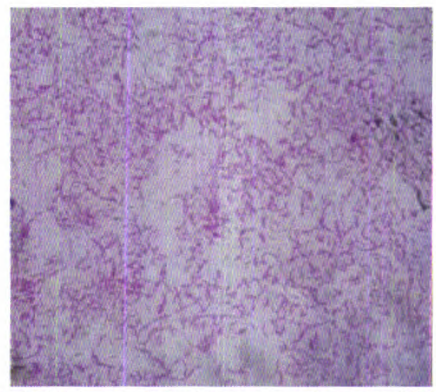


Fig 7.10 Loss of Architecture of tissue (40X)

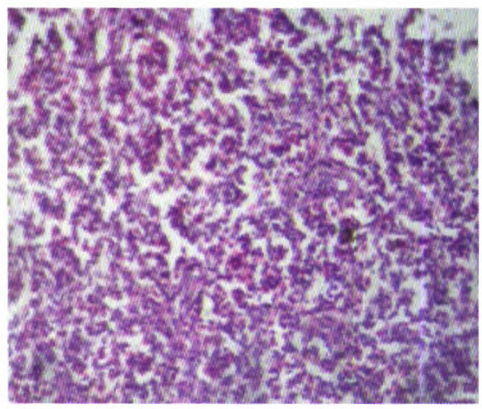


Fig 7.11 Necrosis and Loss of Architecture of tissue (20X)

Histopathological alterations in the liver of *Etroplus maculatus* on exposure to sublethal concentrations of lead nitrate (Haemotoxylin & Eosin)

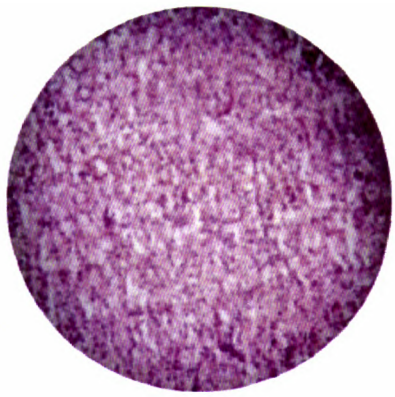


Fig 7.12 Control liver (20X)

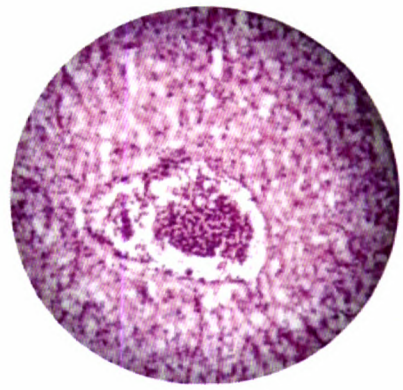


Fig 7.13 Hyalinization20X)

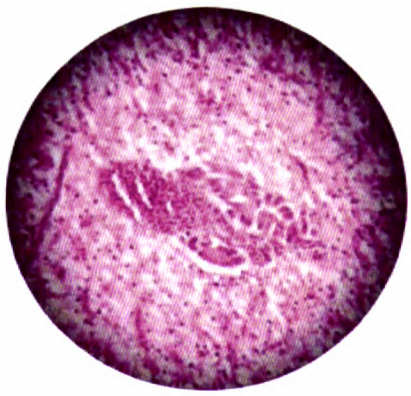


Fig 7.14 Hemorrhage (20X)

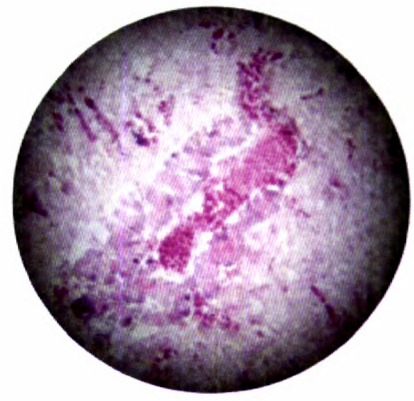


Fig 7.15 Hemorrhage (40X)

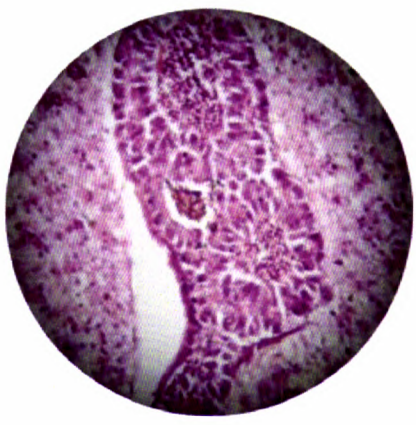


Fig 7.16 Infiltration and blood clots in Hepatocytes (40X)

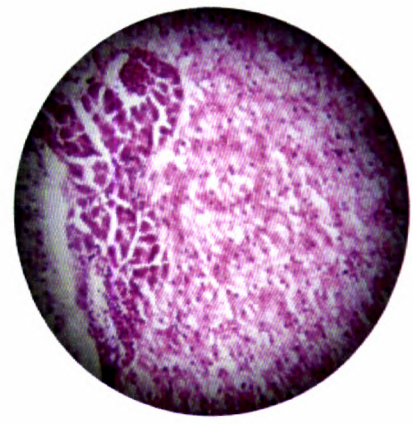


Fig 7.16 Infiltration and Development of Necrotic area (40X)

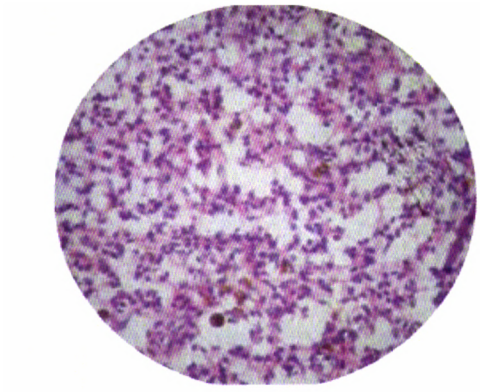


Fig 7.17Necrotic area (40X)

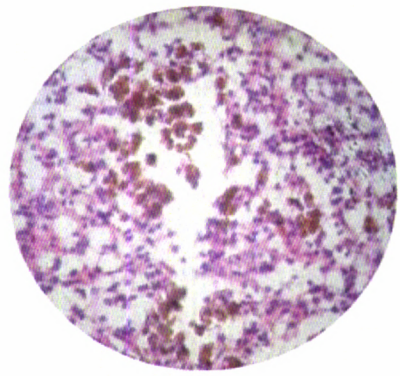


Fig 7.17Necrosis and brown pigmentation(40X)

7.3.2 Gill in control fish

The gill tissue of the control fish has the normal gill structure. The control fish has got four pairs of branchial arches on the pharynx. Two sets of four holo branches arise from the branchial arch forming the sides of the pharynx. Each holo branch consists of two hemi branches 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 holo branchs. Close examination of the hemi branches of gill shows that they consists of a row of long thin filaments, the primary lamellae, which project from the arch like teeth of a comb. The surface area of the primary lamellae is increased further by the formation of regular semi linear folds across its dorsal and ventral surface of the secondary lamellae. The dorsal and ventral rows of secondary lamellae on each primary lamella are staggered so that they complement the spaces in the rows of lamellae of adjacent filaments. Secondary lamellae are flattened and delimited by a simple flat, fine epithelium. The secondary lamellae together with the central vascular spaces form the gaseous exchange barrier or respiratory barrier. The entire structure is supported by pillar cells.

7.3.2.1 Changes in gills for 0.33 ppm and 1.2 ppm of cadmium chloride for 14 th and 28 th day of exposure

At 0.33 ppm of cadmium chloride for 28 days of exposure the changes observed in the gill included atrophy, fusion of gill filaments, marked degeneration and necrosis of the epithelial cells, marked disorganization and rupture of the secondary lamellae. The severity of changes caused by cadmium chloride was

positively correlated with the concentration. The fish gill at the concentration of 0.33 ppm showed enlargement of gills and substantial increase in the number of mucous cells. The epithelial cells of primary and secondary lamellae were swollen and edematous and filed with red blood cells.

At higher concentration of 1.2 ppm of cadmium chloride for 28 days of exposure showed fusion of secondary lamellae and clubbing of secondary lamellae. Lamellar talengiectasis or aneurysm was also observed. Blood vessels were frequently packed with red blood cells especially near the gill filament and necrosis. On the 14 th day of exposure of cadmium chloride disorganization of the branchial tissue, cellular necrosis, hypertrophy of the mucous pavement cell, dissociation and rupture of the branchial epithelia, fusion of the secondary lamellae, swelling of the capillaries (secondary lamellae), dissociation between the epithelium and the pillar cells were observed.

7.3.2.2 Changes in gills observed on exposure to 2 ppm and 6.6 ppm lead nitrate in 14th and 28th day of exposure

On the lower concentration of 2ppm of lead nitrate on the 14 th day of exposure the gills retains its original shape and structure but the spreading of secondary lamellae and lamellar disorganization were common. On the 28 th day of exposure alterations such as blood congestion, hypertrophy of epithelial cells and lamellar disorganization were also observed. Swelling of the secondary lamellae, fusion of the secondary lamellae was common.

At higher concentration of lead nitrate for 14 days of exposure, the changes observed in the gill included epithelial hyperplasia, atrophy and fusion of gill filaments, and marked degeneration and necrosis of the epithelial cells. At higher concentration of lead nitrate for 28 day of exposure, the commonest anomalies found were dilation of the marginal channel, hyperplasia of the epithelial cells and lifting of the lamellar epithelium and rupture of the secondary lamellae. There were some cases where the hyperplasia was more severe, resulting in the fusion of some secondary lamellae. Frequently, alterations such as blood congestion hypertrophy of epithelial cells and lamellar disorganization were also observed.

Histopathological alterations in the gills of *Etroplus maculatus* on exposure to sublethal concentrations of cadmium chloride (Haemotoxylin & Eosin)

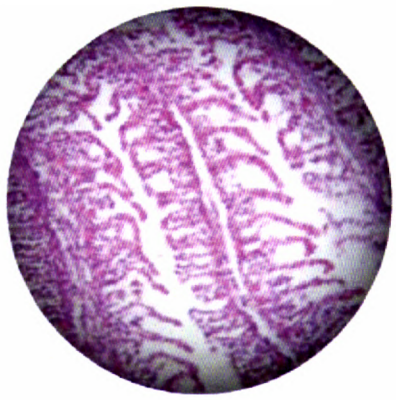


Fig 7.18 Control tissue (40X)

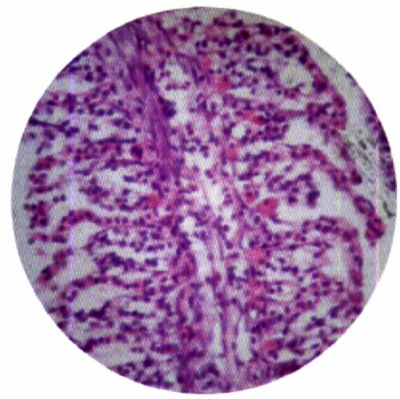


Fig 7.19 Leuckocyte Infiltration (40X)

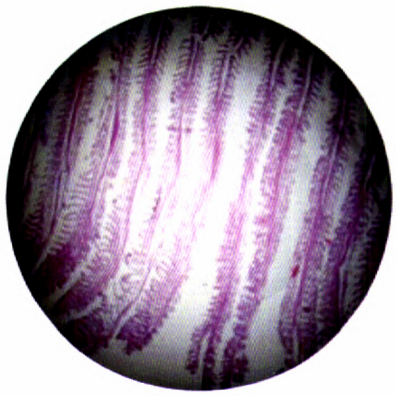


Fig 7.20 Rupture of filaments (40X)

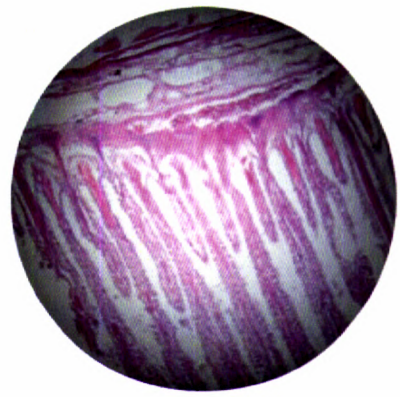


Fig 7.21 Hyperplasia (40X)

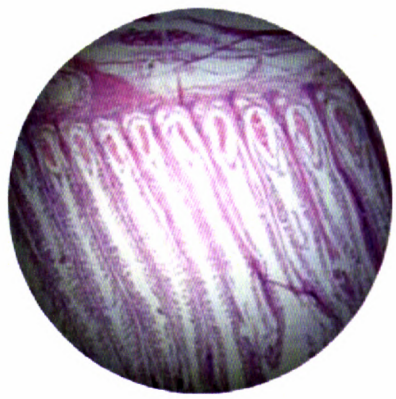


Fig 7.22 Hyperaemia (40X)

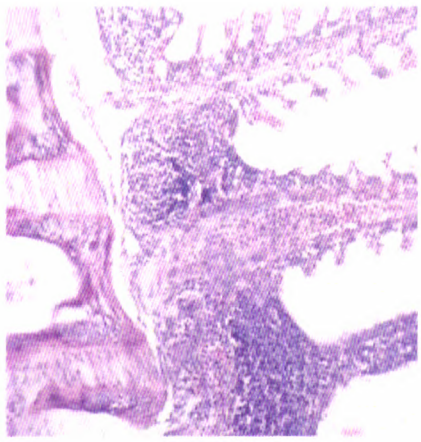


Fig 7.23 Detachment of base membrane (40X)

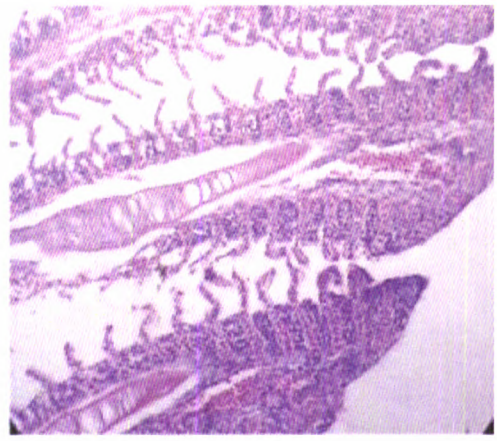


Fig 7.24 Hyperplasia (40X)

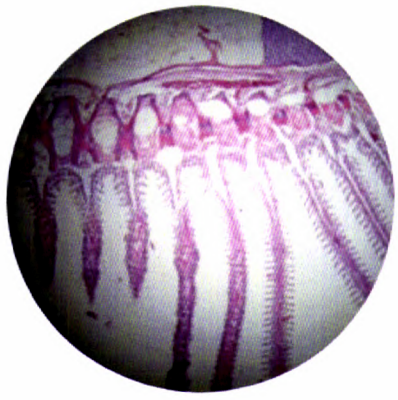


Fig 7.25 Hyperaemia (20X)

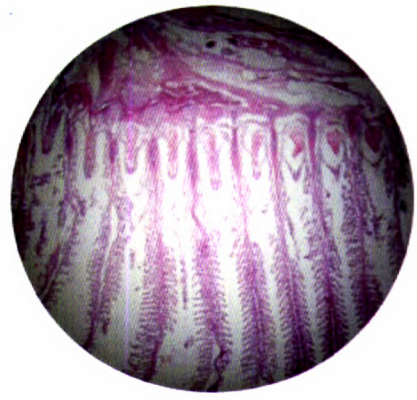


Fig 7.26 Rupture of filaments (20X)

Fig 7.27 Futions of filaments (20X)

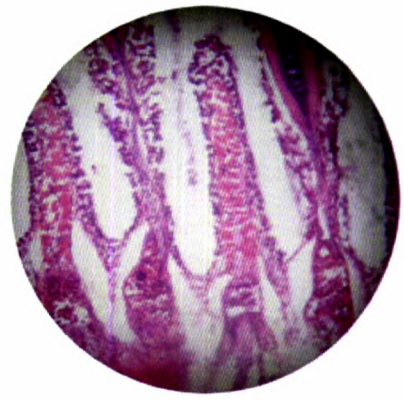


Fig 7.28 Hyperplasia (40X)

Histopathological alterations in the gills of *Etroplus maculatus* on exposure to sublethal concentrations of lead (Haemotoxylin & Eosin)

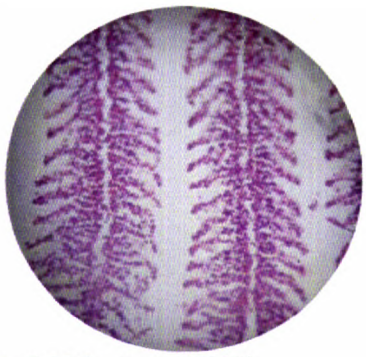


Fig 7.29 Control gil (40X)

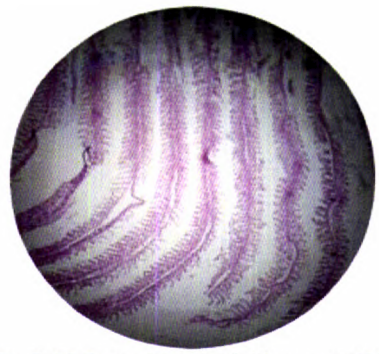


Fig 7.30 Detachment of filaments (40X)

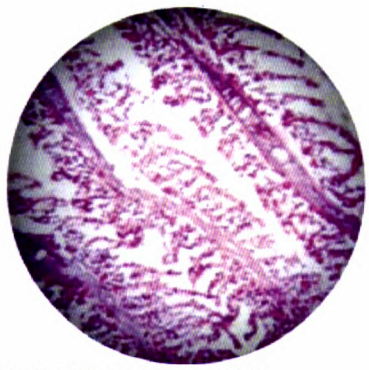


Fig 7.31Hyperplasia (40X)

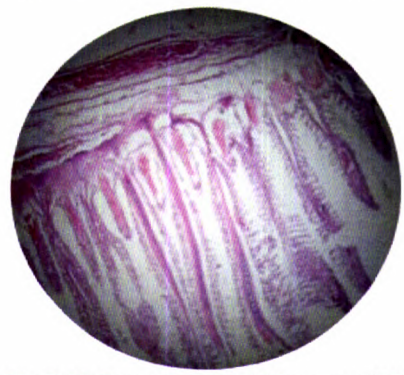


Fig 7.32 Detachment of filaments (40X)

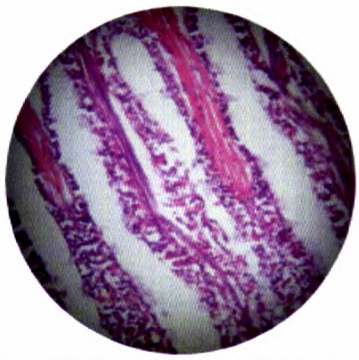


Fig 7.33 Hyperplasia and fusion of secondary filaments(40X)

Fig 7.34Hyperiemia (40X)

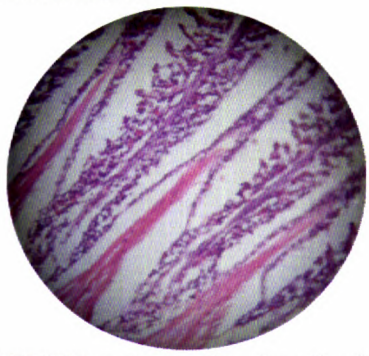


Fig 7.35 Lifting of secondary lamellae (40X)

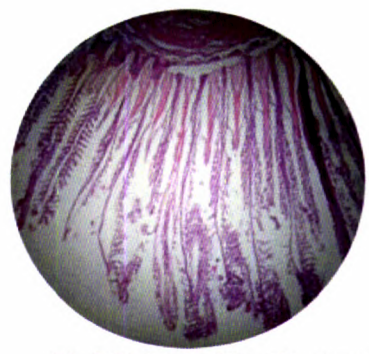


Fig 7.36 Loss of Architecture (40X)

7.3.3 Histopathology of kidney

7.3.3.1Changes in kidney exposed to 0.33 ppm and 1.2 ppm of cadmium chloride for 14 th and 28 th day of exposure

At a concentration of 0.33 ppm of cadmium chloride for 28 th day of exposure there was a decrease in Bowman's space, blood in Bowman's space, hyaline droplets and degeneration were observed. Tubular degeneration, decrease of the tubular lumen- was also seen. On the 14 th day of exposure cloudy swelling and cellular hypertrophy and nuclear hypertrophy occurred.

On the 14 th day at 1.2 ppm of cadmium chloride irregular shaped cells, eosinophilic granules in cytoplasm, melanomacrophages aggregates were observed. Bowman's space decrease, blood in Bowman's space, hyaline droplets degeneration and tubular degeneration, decrease of the tubular lumen were also noticed.

On the 28 th day of exposure to cadmium chloride at 1.2 ppm nuclear hypertrophy, irregular shaped nucleus, nucleus in a lateral position were the common alterations observed and the other alterations were cellular hypertrophy, cytoplasmic vacuolation, and cellular atrophy. On the 14 th day at 1.2 ppm irregular shaped cells, eosinophilic granules in cytoplasm, melanomacrophages aggregates were observed. Bowman's space decreased, blood in Bowman's space, hyaline droplets degeneration, tubular degeneration and decrease of the tubular lumen were also seen.

7.3.3.2 Changes in kidney exposed to 2 ppm and 6.6 ppm of lead nitrate for 14 th and 28 th day of exposure

The alterations found in the kidney are atrophy of glomerulus, glomerular expansion, resulting in reduction of Bowman's space in the tubules; the most frequent alterations were cloudy swelling, occlusions of tubular lumen and hyaline droplet degeneration. Less frequently, regenerating tubules were seen.

Histopathological alterations in the kidney of *Etroplus maculatus* on exposure to sub lethal concentrations of cadmium chloride (Haemotoxylin & Eosin)

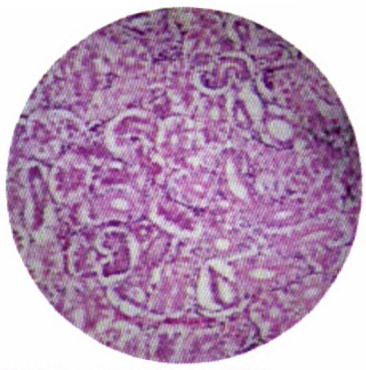


Fig 7.37 Control kidney (40X)

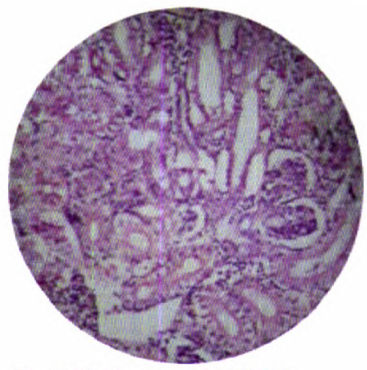


Fig 7.38 Vacuolation (40X)

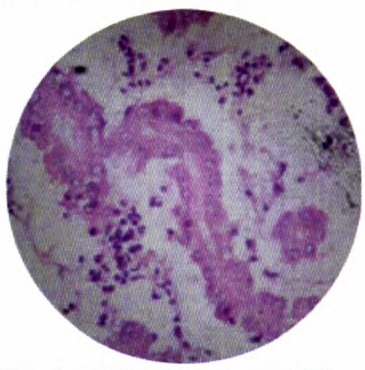


Fig 7.39 Eosinophil Infiltration (100X)

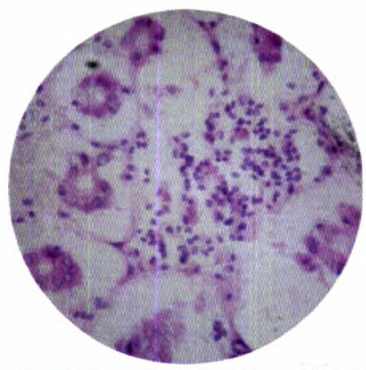


Fig 7.40 Eosinophil Infiltration (100X)

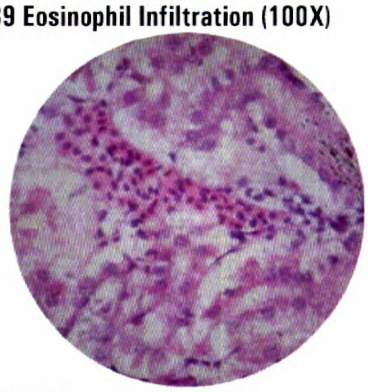


Fig 7.41 Haemorage (100X)

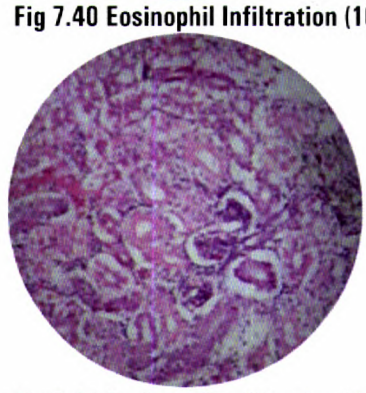


Fig 7.41 Eosinophil Infiltration (100X)

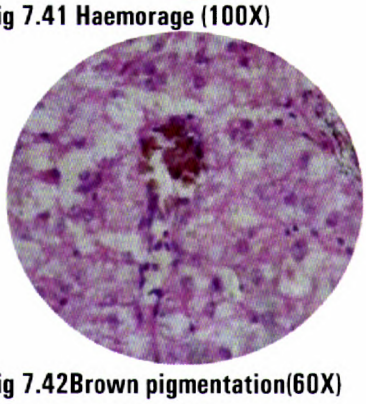


Fig 7.42Brown pigmentation(60X)

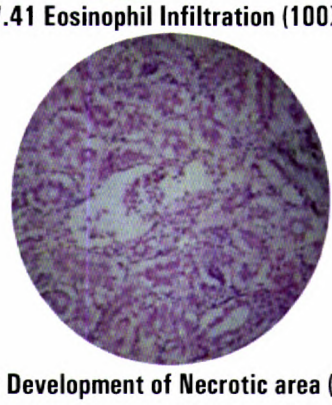


Fig 7.43 Development of Necrotic area (20X)

Histopathological alterations in the kidney of *Etroplus maculatus* on exposure to lead nitrate (Haemotoxylin & Eosin)

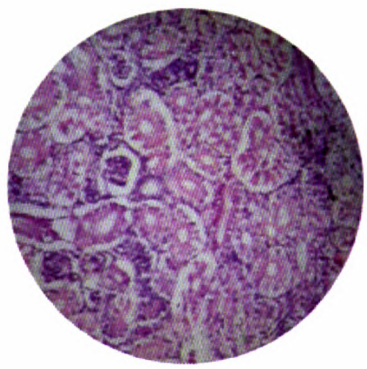


Fig 7.44Control kidney (40X)

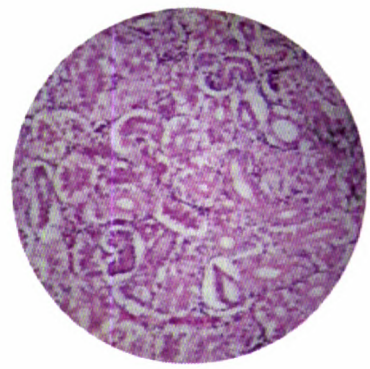


Fig 7.45 Loss of structure (40X)

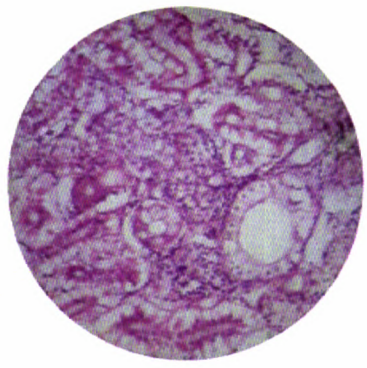


Fig 7.46Enlargement of Boman's capsule(40X)

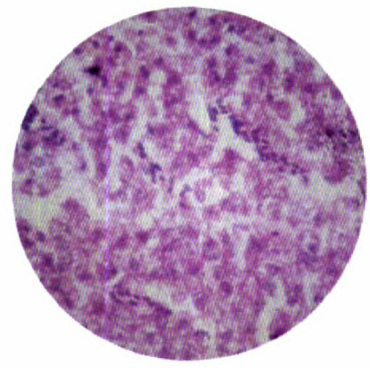


Fig 7.47 Eosiniphil infiltration (40X)

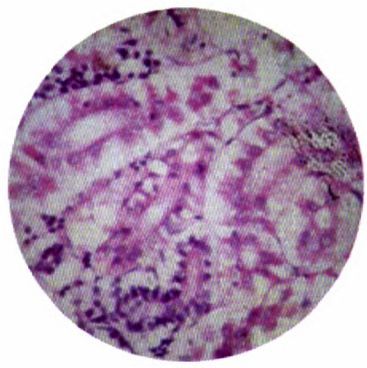


Fig 7.48 Loss of Architecture (40X)

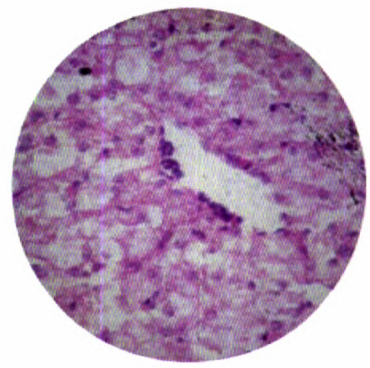


Fig 7.49 Development of Necrotic area (40X)

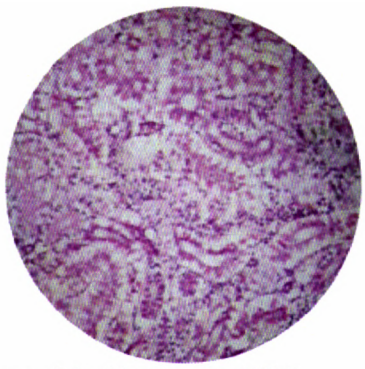


Fig 7.50 Loss of Architecture and High Necrosis (40X)

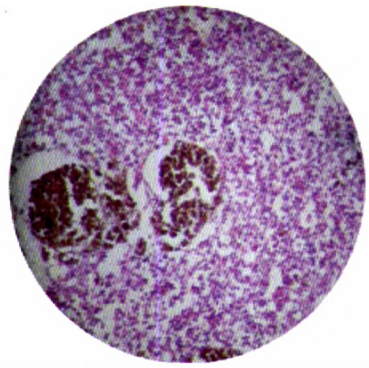


Fig 7.51 Haemosiderin pigmentation (40X)

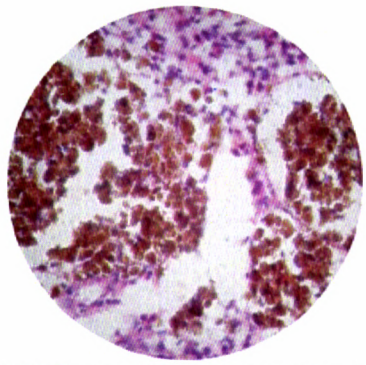


Fig 7.52 Haemosiderin pigmentation (40X)

7.4 DISCUSSION

There is a need for sensitive bio-monitoring tools in toxicant impact assessment to indicate the effect of toxicants on fish health in the aquatic ecosystems. Histopathological assessment gives early warning signs of disease and detection of long term injury in cells, tissues or organs. Histopathology provides a rapid method to detect effects of irritants in various organs (Johnson *et al.*, 1993) and it is a higher–level response, reflecting rly alteration in physiological and biochemical function (Hinton *et al.*, 1992).

7.4.1 Liver

The liver plays a key role in the metabolism and biochemical transformations of pollutants from the environment, which inevitably reflects on its integrity by creating lesions and other histopathological alterations of the liver parenchyma or the bile ducts (Roberts, 1978). The organ associated with the detoxification and biotransformation process is the liver, due to its function, position and blood supply (Van der Oost *et al.*, 2003) is also one of the organs mostly affected by contaminants in the water (Rodriguez and Fanta, 1998).

The liver showed vacuolar degeneration of the hepatocytes around the congested hepatic sinusoids and ventral vein. Nuclear pyknosis is observed in the majority of hepatic cells. Some pancreatic acini suffered degeneration and devoid of zymogenic granules. These findings are in agreement with Samya, (2008). The metal binding proteins were present in the nuclei, sinusoids, extracellular space and the acinar cells of hepatopancreas suggested that the increase in the cell damages (De Smet and Blust, 2001). Similar results were observed by Van Dyk (2003) and Mela *et al.*, 2007).

Vacuolation of hepatocytes reported in cadmium exposure in the said to be associated with the inhibition of protein synthesis, energy depletion, disaggregation of microtubules or shift in substrate utilization were supported by Hinton and Lauren, (1990). Congestion of centrolobular veins and presence in their proximity of some hyalinized areas as a result of hepatocyte degenerescence is another often meted aspect. Hyalinization seems to be the result of disturbances of protein synthesis reported by Van Dyk *et al.*, (2007). All this findings are in agreement with the observations of Triebskorn *et al.*, (2008) and Nicula Marioara *et al.*, (2009).

The liver parenchyma of the animals exposed to cadmium showed signs of cytoplasmic and nuclear degeneration and nuclear vacuolation besides the focal necrosis that was also found in cadmium treated fish. These alterations are more severe and have been associated with the exposure of the fishes to contamination by metals, such as copper (Paris-Palacios *et al.*, 2000) and mercury (Oliveira Ribeiro *et al.*, 1996), and by polychlorinated biphenyls (PCBs) (Chang *et al.*, 1998).

The presence of bile stagnation and melanomarophages in great quantity in the livers of *Etroplus maculatus* exposed to sublethal concentrations of cadmium and lead showed strong evidence that these organs suffered structural and metabolic damage due to exposure to the heavy metals.

The microscopic analysis of the histological preparations indicated presence of hemorrhaging in the hepatocellular, parenchyma; moreover the pigment hemosiderin was evidenced, probably as a result of internal bleeding in the hepatic tissue of Barbel. Hemosiderin in the liver is represents a product of hemoglobin degradation that has been filtered out by the lymphoid–macrophage system (Khan *et al.*, 1994.)

In general, fish livers contained hemosiderin pigments that may result from destruction of erythrocytes. Break down of hemoglobin converting into hemosiderin was responsible for the brown deposits with in the hepatic tissues (Fayed, 2004). Several factors have been held responsible for the abnormal accumulation of hemosiderin in the liver tissue to be rapid and continue destruction of erythrocytes with increased hemolysis and damage of the iron metabolism.

The present study shows that the histopathological changes in the liver cause metabolic problems as well. Evidence for this is the bile stagnation in liver of most fish studied. This lesions, characterized by the remains of the bile in the form of brownish-yellow granules in the cytoplasm of the hepatocytes (Pacheco and Santos, 2002), indicates that the bile is not being released from the liver. This accumulation of bile indicates possible damage to the hepatic metabolism (Fanta *et al.*, 2003).

Anomalies such as irregular shaped hepatocytes, cytoplasmic vacuolation and nucleus in a lateral position, close to the cell membrane, were observed in the siluriform *Corydoras paleatus* contaminated by organophosphate pesticides(Fanta *et al.*, 2003). Vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver. Depletion of the glycogen in the hepatocytes is found in stressed animals (Hinton and Lauren, 1990; Wilhelm Filho *et al.*, 2001), because the glycogen acts as a reserve of glucose to supply the higher energetic demand occurring in such situations (Panepucci *et al.*, 2001).

Pacheco and Santos (2002) described increased vacuolization of the hepatocytes as a signal of degenerative process that suggests damage, possibly related to exposure to contaminated water. An increase in the density of the melanomacrophage aggregates, as observed in the liver of *Etroplus maculatus* in the study , is generally related to important hepatic lesions (Pacheco and Santos, 2002), such as degenerative and necrotic processes. This was related, in *Pleuronectes americanus*, to contamination with Polycyclic aromatic hydrocarbons and pesticides in urban areas on the USA coast (Chang *et al.*, 1998). The function of the melanomacrophages in the liver of fishes remains uncertain, but some studies have suggested that it is related to destruction, detoxification or recycling of endogenous and exogenous compounds (Haaparanta *et al.*, 1996). Aly *et al.*, (2003) reported vacuolar degeneration and necrosis and hemolysis of red blood cells in the liver of *Clarias gariepinus* on exposure to lead pollution.

7.4.2 Gills

The gills are the largest proportion of the outer surface area of fish and a few micrometers separate the blood from the water (Wood and Soivio, 1991), facilitates gaseous exchange. The structure and dimensions of fish gills are important parameters with which to assess their functions (Laurent, 1982). Gills are organs for respiratory gas exchange, osmoregulation, excretion of nitrogenous waste products and acid base regulation, are directly affected by contaminants. The substantial surface area of the gills in fish serves as an interface between the environment and blood notably for the continuous diffusion of oxygen and the maintenance of acid–base and ion balance (Randall *et al.*, 1996; Claiborne *et al.*, 2002). Exposure to environmental contaminants leads to significant tissue alterations in the gills.

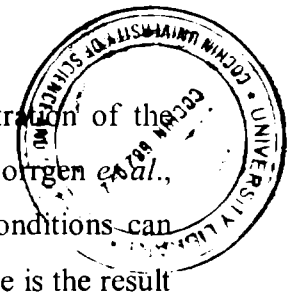
The immediate morpho-pathological response of the gills of fish exposed to ambient xenobiotics (including metal salts) is often manifested by a significant increase in the density of its mucous cells. The large quantity of mucous secretion acts as a defense mechanism against several toxic substances (Sellers *et al.*, 1975; Mc Donald, 1983; Handy and Eddy, 1991; Mazon *et al.*, 1999). The regular sloughing of mucus from the surface of gills into the media helps to remove the bound pathogens, toxicants and foreign matters (Powell, *et al.*, 1992) including

metal compounds (Lock and Vanoverbeeke, 1981) which adheres to the gills. The mucous coat covering the gill epithelia is composed of mainly of glycoproteins that have electro negative charges. It is perhaps due to the well established ability of these glycoproteins to trap heavy metal ions (Lock and Vanoverbeeke, 1981).

According to Daoust *et al.*, (1984) exposure to heavy metals very often alters the chemical composition or thickness of mucous layer may disturb the normal ability to recognize different cell types. This is due to contact stress and may also due to transformation of electrically charged properties of the epithelial cells which favor adhesion between the cells of neighboring secondary lamellae, which has been a very common manifestation of the toxic impact of a large number of xenobiotics including lead salt. Several other xenobiotics are also known to induce fusion of the secondary lamellae of gills (Leino, *et al.*, 1987; Dutta *et al.*, 1996; Wendelaar Bonga, 1997).

The present observation in the presence of mucous in the ballooning dilation in the treated gill filaments may be considered as an ion trap to concentrate trace elements from water and favor cell adhesion between the neighboring secondary lamellae. It may as well serve to protect the epithelia against both mechanical abrasion and infection as suggested by Olson and Fromm, (1973) and also the presence of white cells, mainly macrophage, lymphocytes and neutrophils clearly indicates an inflammatory reaction and cellular infiltration thus reflecting an immunological response of the fish to environmental contaminants (Zeeman and Brindley, 1981; Tao *et al.*, 2000).

Bhagwant and Elahee (2002) observed various gills tissue modifications in two fish species *Mulloidichthys flavolineatus* and *Mugil cephalus* treated in the effluent collected from the Bay of Poudre d'Or in Mauritius containing effluent discharge pollutants. The tissue modifications are displacement of the epithelial layer of the secondary lamellae from the underlying connective tissue. The epithelial lifting is often accompanied by oedematous condition. Cellular hyperplasia, ballooning formation at the epical end of secondary lamellae, deformities and stunting of secondary lamellae. Cellular damage along with circulatory anomalies gives rise to most severe gill lesion known as teleangiectasia.



The severity of damage to the gills depends on the concentration of the toxicant and the period of exposure (Oliveira *et al.*, 1996; Karlson-Norrgen *et al.*, 1985; Mallat, 1985; Franchini *et al.*, 1994). Toxic environmental conditions can result in two types of structural changes in tissues of the organism. One is the result of the direct toxic effect of the pollutant leading to degeneration and necrosis. The second is a result of compensatory mechanisms that deal with the environmental stressor, as in cellular hyperplasia (Hughes and Perry, 1976). The compensatory changes become maladaptive if the duration of the stress factor exceeds the biological tolerance limits (Wedemeyer, *et al.*, 1990). The teleangiectasia observed in fishes may affect blood circulation leading to respiratory impairement. This type of structural damage shows close similarity to lesions brought about by elevated levels of other environmental pollutants such as Zinc (Skidmore and Tovell, 1972), ammonia (Smart, 1976) and sediment-borne contaminants (Hargis Jr. and Zwerner, 1988). The white cell infiltration, displacement of epithelial cells and edema could contribute to an increase in the diffusion distance from surrounding water to capillaries and simultaneously increase the amount of tissue in the secondary lamellae. This hypertrophy could result in a decrease of the respiratory dead volume between the lamellae and impair the diffusion of oxygen through the swollen epithelium.

An increase in the lamellar dense cells in the secondary lamellae of brook trout exposed for a long to acid and aluminium was previously reported by Tietge *et al.*, (1988). The fusion of the secondary lamellae could cause a decrease in free gas exchange, thus affecting the general health of the fish (Skidmore and Tovell, 1972; Gardner and Yevich, 1970)

Nile tilapia cultured in waste water polluted with cadmium, lead, zinc, Iron and manganese showed mild congestion and edema of the primary lamellae, severe hyperplasia, fusion and focal desquamation of the epithelial lining of the secondary lamellae, gill arch showed numerous mononuclear leukocytic infiltration, edema and congestion (Samya, 2008).

The histological alterations attributed to prolonged exposure to heavy metals lead to respiratory, osmoregulatory and circulatory impairment. These findings

were demonstrated by Fernandes *et al.*, (2008). Moreover Alvarado *et al.*, (2006) reported that, dramatic increase of chloride cells in the gills that produces epithelial thickening of the filament epithelium enhances migration of chloride cells up to the edge of the secondary lamellae and provokes the hypertrophy and fusion of secondary lamellae.

Weber (1991) observed after a 30 day lead exposure caused, intralamellar hyperplasia and increased mucus secretion in gill filaments. Cumulatively, lowered oxygen uptake and transport abilities, and ion exchange capacities at gill surfaces (Tabche *et al.*, 1990) could result in decreased swimming capacity. Yet, another explanation for decreased swimming capacity is lead induced central nervous system dysfunction. The medulla a critical site for respiratory control, contains both site for respiratory control, contains both cholinergic and catecholinergic nuclei that control such functions as mucus secretions on the gill surface, opercular stroke frequency, gill and systemic blood vessel vasoconstriction, and heart stroke (Lagler *et al.*, 1962; Randall, 1970; Butler and Matcalfe, 1983; Smith, 1984). Lead interferes with selected muscarinic receptors in the brain and may be an important mechanicsm for some leasd induced behavoiural alterations (Costa and Fox, 1983; Schulte *et al.*, 1994; Cory – Slechta and Pokora, 1995)

Gill lesions in *Etroplus maculatus* subjected to lead nitrate poisoning are characterized primarily by marked hyperplasia of epithelial cells in the secondary lamellae and the intra cellular space and by severe necrosis in both structure. These findings are in agreement with the experimental lead nitrate poisoning in the gills of Tinca tinca (Roncero *et al.*, 1990). Primary lesion in secondary lamellae was à severe edema was located principally in the distal part of the lamellae although other authors report a clearly basal location.

Lesions observed in secondary lamellae of the gills at all stages of the experiment resembled those reported by other authors. (Roberts, 1982; Rojik *et al.*, 1983). Edema, epithelial hyperplasia and necrosis gave rise to acute respiratory distress. This coupled with the reduction in surface area of the respiratory barrier of secondary lamellae, observed throughout the experiment, and the inhibition of

enzyme mitochondrial transport systems led to the inevitable death of the fish (Rojik *et al.*, 1983; Crespo and Sala, 1986).

Most part of the gill lesions caused by sub lethal exposures affects lamellar epithelium (Hinton and Lauren, 1990); however, some alterations in blood vessels may also occur, when fishes suffer a more severe type of stress. In this case, damaged pillar cells can result in an increased blood flow inside the lamellae, causing dilation of the marginal channel, blood congestion or even an aneurysm (Takashima and Hibiya, 1995; Rosety-Rodriguez *et al.*, 2002). The present work observed the formation of an aneurysm is related to the rupture of the pillar cells (Heath, 1987; Martinez *et al.*, 2004) due to a bigger flow of blood or even because of the direct effects of contaminants on these cells. This is a severe type of lesion, recovery from which is possible, but more difficult than the epithelial changes (Poleksic and Mittrovic –Tutundzic, 1994).

In the present study there was swelling of the secondary lamellae evidenced by changes to both the lamellar region and increased volume of tissue lying outside the pillar system. Additionally, decreased lamellar height and increase lamellar width indicated a reduction in lamellar surface area available for gas diffusion. Mallat (1985) stated that reflecting direct toxic action, lifting and hyperplasia of the lamellar epithelium could be interpreted as defense responses of the fish, as these alterations increase the distance across which waterborne irritants must diffuse to reach the blood stream. Epithelial lifting and hyperplasia of undifferentiated epithelial cells are known to be nonspecific alterations, which can be caused by a variety of unrelated insults such as those caused by heavy metals (Heath, 1987; Hinton *et al.*, 1992; Randy *et al.*, 1996)

The teleost fish gill covered by a complex epithelium whose function is regulated by perfusion through an intricate vascular system. In addition to being the site of gas exchange for these aquatic animals, the gill epithelium possesses transport steps which mediate active and passive movements of ions, counteracting dissipative movements down electrochemical gradients between the fish's blood and water. These same transport steps play major roles in acid-base regulation and excretion of unwanted nitrogen in the form of ammonia. A variety of aquatic

pollutants produce gross histopathological changes of the gill epithelium, which are often associated with osmoregulatory, acid-base, or hemodynamic malfunction. Since similar pathways and receptors are common to a variety of human tissues, which are affected by environmental pollutants (eg. kidney, intestine, liver, blood vessel etc.), the fish gill presents an apt model which may be used to examine general pathologies induced by toxic substances.

Epithelial necrosis, secondary lamellae showing fusion and lifting of epithelium have also been showed in other species (Cengiz and Unlu, 2002, 2003). Alterations like epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae are examples of defense mechanisms., since, in general, these result in the increase of the distance between the external environment and the blood and thus serve as a barrier to the entrance of contaminants (Mallatt, 1985; Hinton and Lauren, 1990; Poleksic and Mitrovic-Tutundzic, 1994; Fernandes and Mazon, 2003). Engelhardt *et al.*, (1981) observed epithelial lifting and lamellar fusion in Rainbow trouts (*Oncorhynchus mykiss*) exposed to petroleum residues. Similar alterations in the gills have also been reported in the fishes exposed to metals (Oliveira Ribeiro *et al.*, 2000; Cerqueira and Fernandes, 2002; Martinez *et al.*, 2004) and organic contaminants (Rosety-Rodriguez *et al.*, 2002; Fanta *et al.*, 2003). According to Mallat (1985) such alterations are non-specific and may be induced by different types of contaminant (Mallat, 1985). As a consequence of the increased distance between water and blood due to epithelial lifting, the oxygen uptake is impaired. However, fishes have the capacity to increase their ventilation rate, to compensate low oxygen uptake (Fernandes and Mazon, 2003).

The multilayered epithelium of the interlamellar space was also affected (Fowler, 1987; Roberts, 1982; Rojik *et al.*, 1983; Crespso and Sala, 1986). Necrosis was observed over the latter half of the experiment, becoming severe in the last batch. Epithelial cells and chloride cells were the worst affected. Lead caused cell hyper activity, with the resulting proliferation of chloride cells and substantial increase in ion exchange (Crespo and Sala, 1986), leading subsequently to

degeneration and necrosis of cell elements and severe alteration in the osmotic regulation of the internal medium.

Toxic substances can injure gills, thus reducing the oxygen consumption and disrupting the osmoregulatory functions of aquatic organisms (Saravana *et al.*, 2000). In the present study, gills were found to be the most seriously affected organ compared to liver, kidney, and spleen perhaps because of the direct contact with the compound. Liver has the ability to degrade toxic compounds, but its regulating mechanisms can be overwhelmed by elevated concentrations of these compounds, and could subsequently result in structural damage (Brusle *et al.*, 1996).

Gill has an extensive surface area and minimal diffusion distance between dissolved O_2 and blood capillary for efficient gaseous exchange. This organ remains protected by a thin layer of mucous coating (Hughes *et al.*, 1979; Powell, *et al.*, 1992). Electron microscopic investigations have shown that the surface of the gill epithelia is provided with numerous micro-ridges which anchor the mucus film (Hughes and Wright, 1970). The number and pattern of the micro ridges are disturbed following treatment of the fish with various stress conditions, including exposure to heavy metals, may diminish the capacity of gas exchange by reducing both the lamellar area and micro-turbulence (Karlson- Norrgren *et al.*, 1986a ; 1986b).

Mallat (1985) and Novak (1992) and Fanta *et al.*, (1995); have reported that the most common effect of any pollutant or water quality change in secondary lamellae is the lifting of it which leads to large increase in the diffusion distance and thereby to less oxygen consumption. Stentiford *et al.*, (2002) reported that the gill epithelium is a major route for the uptake of soluble xenobiotics by fish and he observed the highest prevalence of cellular hypertrophy, hyperplasia and fusion of the secondary lamellae in flounder caught from the polluted estuaries.

7.4.3 Kidney

The teleostean kidney is one of the organs to be affected by contaminants in the water (Thophon *et al.*, 2003). Most common alterations found in the kidney of fishes exposed to water contamination are tubule degeneration (cloudy swelling and

hyaline droplets) and changes in the corpuscle, such as dilation of capillaries in the glomerulus and reduction of Bowman's space (Takashima and Hibiya, 1995). Exposure to metals frequently causes alterations in the tubules and glomerulus, such as described by Thophon *et al.*, (2003) for the perch (*Lates calcarifer*) exposed to cadmium; Handy and Penrice (1993) found swollen Bowman's capsule cells and melanomacrophages in the kidney of trout (*Salmo trutta*) and tilapia (*Oreochromis mossambicus*) exposed to mercuric chloride. Similar alterations were found in fishes exposed to organic contaminants (Veiga *et al.*, 2002) and mixed environmental; contaminants (Schwaiger *et al.*, 1997; Pacheco and Santos, 2002). These reports suggest that the histopathological changes in the kidney, like in gills, could not be considered specific to the stressors. In the present study, kidney of the fish often showed cloudy swelling in tubule cells. This alteration can be identified by the hypertrophy of the cells and the presence of small granules in the cytoplasm, which takes on the appearance of a net. This initial stage in the degeneration process can progress to hyaline degeneration, characterized by the presence of large eosinophilic granules inside the cells. These granules may be formed inside the cells or by the re absorption of plasma proteins lost in the urine, indicating damage in the corpuscle (Hinton and Lauren, 1990). In more severe cases, the degenerative process can lead to tissue necrosis (Takashima and Hibiya, 1995).

Saxena and Saxena (2008) reported that congestion and hemorrhages in kidney, engorged blood vessels and extravagated erythrocytes could be seen in tissue sections of sampled kidney of fish after exposure to water polluted with heavy metals. Moderate lymphocytic infiltration was observed in kidney. Aggregates of round cells with large blue staining nuclei were clearly visible. Kidney showed degenerative changes in the parenchyma. Cellular structure of tissues was changed and homogenous structure of tissues was lost in the liver and kidney of treated fishes.

The development of new nephrons occurs only in the neonatal period in mammals, while, in fish this process continues throughout life, being more frequent in young and fast growing fishes (Reimschuessel, 2001). Hinton and Lauren (1990) and Cormier *et al.*, (1995) have reported the increase in the frequency of new

nephrons and regenerated tubules, during the process of the recovery of the damaged kidney in fish. Similar observation have also been made in the siluriform *Ameiurus nebulosus* and in cod (*Microgadus tomcod*) collected from contaminated streams (Cormier *et al.*, 1995) , and in gold fish (*Carassius auratus*), rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Brachidanio rerio*) and tilapia (*Oreochromis mossambicus*) exposed to contaminants such as mercury, antibiotics and solvents, that are known to cause necrosis and vacuolization (Reimschuessel, 2001). The short period of exposure may have not been sufficient to start the regeneration process, because this phenomenon usually starts from 2 to 4 weeks after exposure to the stressor (Reimschuessel, 2001), and could even take 2 months to be completed (Gernhofer *et al.*, 2001).

There is evidence that lead has endocrine–disrupting potential. Short-term and life cycle tests with laboratory fish species are used to identify hazards associated with the discharge of endocrine–disrupting chemicals (EDCs) into the environment through a suite of biomarkers of exposure to EDCs, including histopathological changes and molecular endpoints (Gray and Foster, 2003).

7.5 CONCLUSION

Histological examination of tissue is an essential step in determining the effect of xenobiotics on fish diagnosing fish diseases. However, pathological lesions for specific disease or toxicant are rare especially when examining fish from wild, because in reverie system where interaction of various pollutants occur as extension of pollution level fluctuates and where the system has inherent quality of retrieval. The alterations in histopathological lesions in such ecosystem although detrimental to fish health do not cause death because pollution level becomes sub lethal. This sub lethal pollution logically contributed either to alarm reaction or to secondary response of adaptive stress syndrome. As a conclusion, the findings of the present investigations demonstrate a direct correlation between heavy metal exposure and histopathological disorders observed in tissues. When these pathological endpoints are measured in combination with other parameters like enzyme responses, haematological parameters, a clear picture of the complex interactions between anthropogenic and natural environmental modifiers will emerge.

Chapter 8
Conclusion

CONCLUSION

The use of hematological parameters in diagnosing the health condition of fish is acquiring acceptance worldwide, as a tool in the management of fish farms. Their haematology provides an important tool in the evaluation of its physiological status, reflecting the relative health of the aquatic ecosystem. Haematological studies on fishes have assumed greater significance due to the increasing emphasis on pisciculture and greater awareness of the pollution of natural fresh water resources in the tropics. Such studies have generally been used as an effective and sensitive index to monitor physiological and pathological changes in fishes

Interpretation of fish haematological data is quite difficult due to internal and external variation. It is well known that blood sampling, laboratory techniques, seasonal variations, size and ontogeny of habitat, genetic properties, sex, population density, geographical dispersion, lack of food supply and stress, PH, water and transportation affect haematological data

Growth is another important factor to be taken into account when considering normal values. In proportion to the physical phenomenon of increasing weight, many of the clinical parameters also change which can be measured using advanced methods. The assessment of normal standard norms for clinically healthy fishes at various stages is necessary to identify and assess whether there is any stress of starvation existing among fishes in a pond.

The primary haematological parameters which include haemoglobin concentration, haematocrit value, erythrocyte (RBC) counts and the erythrocyte sedimentation rate (ESR) have been established based on the 50 fish specimens of *Etroplus maculates*. The use of haematological values in disease diagnosis and nutritional status of fish has been documented. Knowledge of the various

parameters is beneficial to the fish farmers, scholars and further development of the fishing industry in the country. The baseline data presented by this study on the haematological indices of *Etroplus maculatus* will contribute to the comparative studies on fish haematology and aspects of fish physiology. It is apparent from the results that for *Etroplus maculates* normal haematological parameters vary at different times of the year and the variation depends on the body weight, physiological condition of the fish, temperature and season. Occurrence of immature erythrocytes is a common feature of fish blood. Erythroplastis were usually seen during the breeding period though the reason is not known

The variation in methodology used for haematological studies, instant changes in physical and chemical properties of micro-environment in which fish lives makes comparison of literature and the establishment of limits for haematological data difficult. Therefore, we propose separate data collection and comparison from healthy and unhealthy fish and also between sexes to obtain further haematological data. Haematological examinations, and the correct interpretation of the results, are becoming of increasing importance in fish farming activities, and their routine utilization is highly recommended as a practical tool to "get to grips" with implementing standard diagnostic procedures in investigating problems associated with fish diseases and parasites. As haematology assessment is gradually becoming routine practice for intensively bred fish, since intensive aquaculture needs accurate information for identification and control of stress situations and/or diseases in order to ensure healthy fish, the evaluation of blood parameters may be quickest way to detect these symptoms. There for, there is an urgent need reliable normal database to be available for species of economic importance.

Environmental pollution is considered as a side effect of modern industrial society. The increasing awareness of the magnitude of environmental problems triggered by human intervention has served to focus attention on the urgent need for sensitive and precise diagnostic tools with a predictive capability for environmental impact assessment. Public concern for this gross pollution has generated a global demand for initiating regulatory measures to control estuarine and near shore

pollution. Pollution has to assume such gigantic proportions that it has become virtually impossible to plan its total eradication. A scientific management of the hazard alone seems feasible. Statutory pollution management guidelines would have to be based on sound scientific advice emerging out of systematic, quantitative and definitive assessment program.

To be useful in practical and routine monitoring, biomarkers selected should be ecologically relevant so that prediction of environmental changes can be made. They should also be sensitive and responsive to environmentally realistic concentrations and preferably exhibit a good dose response relationship to levels of pollution. The responses should be specific and not profoundly affected by confounding factors. Sampling and analysis should be relatively simple and not technically demanding, so that scientific information for environmental management can be collected in a cost effective manner. Except for kidney histopathology, gill and liver histopathologies are highly sensitive to pollutants exposure, and certain hepatic lesions in fish have been well correlated with contaminat exposure.

As haematology assessment is gradually becoming routine practice for aquaculture field and it needs accurate information for identification and control of stress situations and or / diseases in order to ensure healthy fish , the evaluation of blood parameters may be the quickest way to detect these systems.. There fore, there is an urgent need of reliable normal databases to be available for species of economic importance. Moreover, other complemental studies will be done in this species, such as ultrastructure for concluding compilation of reference data for this orange chromide.

Leukocytes are the primary line of immunological defense and in healthy *Etroplus maculates* and therefore, these two granulocytes constitute the primary barrier of the immune system for this species. .Hence, this immune function might be reflected by the lymphocytes and neutrophilic counts, and then a lower number of these cells might result in a less efficient functioning of the immune system. Further studies are needed to improve our understanding of the functional role of fish thrombocytes in defense mechanisms.

The aim of stringent health control values is to provide a fish pathologist with a choice of sensitive methods to enable early detection of physiological deviations from standard form, which may signal the on come of diseases. The numbers, and proportions of leukocytes, and the thrombocytes (organic defense cell) in circulation provide an important representation of defense cell distribution throughout the body. Thrombocytes are considered to have the additional function of haemato-plug formation during blood clotting. These properties reflect the effective immune mechanism, habit, habitat, evolutionary position and especially, the highly adaptive nature of this fish. Further studies are needed to improve our understanding of the functional role of fish thrombocytes in defense mechanisms.

Aquatic toxicology is relatively a new and still evolving displine, originating from the concern for the safety, conservation and protection of aquatic environments, and also as an off shot of toxicology, since degradation of water mass is caused mainly ny anthropogenic toxicants dumped into the water mass is caused mainly by human activities, directly or indirectly. Contamination of aquatic environment by heavy metals whether as a consequence of acute and chronic events constitutes additional source of stress for aquatic organisms. Sublethal concentrations of toxicants in the aquatic environment will not necessarily result in outright mortality of aquatic organisms. The toxicants and pollutants have significant effects, which can result in several physiological dysfunctions in fish. Dysfunction in the fish induces changes in blood parameters possible as a result of blood water content.

Like other heavy metals, cadmium and lead is manifestly accumulative and can not be excreted. Therefore, the consumption of species that have already accumulated quantities of cadmium and lead into their structures leads to higher rates of lead pollution, which continue to rise further up the food chain, reaching their maxima in predators.

The heavy metal toxicity seriously impairs various metabolic functions of the fish *Etroplus maculates*, reflected as alterations in various biochemical constituents. AAT, ALAT ACP and ALP activity in plasma which are conventional indicators of liver injury are observed to be increased in the heavy metal exposed

Etroplus maculates. Plasma activity concentrations of AAT and ALAT are the most commonly used biochemical markers of hepatocellular necrosis. The increase of biomarker enzymes in plasma might be due to the necrosis of liver. The result revealed that heavy metals cadmium and lead affects the intermediary metabolism of *Etroplus maculates* and the assayed enzymes can work as good biomarkers of heavy metal contamination. The present data confirm that AChE to be a sensitive enzyme marker. Acetylcholinesterases, due to its rate of hydrolysis towards the substance acetylcholinesterase, due to its high rate of hydrolysis towards the substrate acetylcholine chloride, confirm its presence in *Etroplus maculates*. It is highly sensitive and is recommended as a useful biomarker in biomonitoring studies.

The apparent sensitivity of ACP and ALP exhibited through fluctuating activity patterns suggests that analysis of these enzymes at different time periods can be used as biomarkers in metal pollution. In agreement with the observations of other workers, the data obtained once again confirm the reliability of choosing AChE activity pattern as a enzyme marker to assess metal stress.

Changes in the chemical composition of natural aquatic ecosystems can distress the non-target organisms, predominately fish. Fish have been largely used to evaluate the quality of aquatic systems as bioindicators for environmental pollutants. In polluted areas, exposure of fish to xenobiotics leads to interactions between these chemicals and biological systems which give rise to biochemical disturbances.

Energy and metabolic status of the fish demonstrates that heavy metals has induced biochemical alterations and caused significant metabolic and physiological consequences. Reduction in the protein content of fish can be due to the rapid utilization of protein due to heavy metal induced stress. The observed decrement in total carbohydrate and glycogen content of the fish and subsequent rise in the blood glucose level indicates that carbohydrates were used to meet demand of the body under stress.

The biochemical changes induced by heavy metals stress is due to disturbed metabolism manifested as inhibition of enzymes, retardation of growth, damage and

dysfunction of the tissues. The increase of biomarker enzymes in plasma might be due to the necrosis of the liver. The assayed enzymes can work as swift and sensitive biomarkers, for monitoring the impact of heavy metal on aquatic biota and eventually entire ecosystem.

The present study reveals that heavy metals has profound effect on the haematological parameters of *Etroplus maculates*. Decline in WBC, RBC and HB values and increase in PCV of the heavy metal treated fish raises a serious impact on the immunity of the fish. The alterations may be disruptive to the survival capacity of the catfish in their natural environment.

Histopathological evaluation points out that exposure to sub lethal concentrations of heavymetals cadmium and lead caused destructive effect in the gill, liver and kidney tissues of *Etroplus maculates*. Liver of heavy metal treated animals exhibited various histopathological features such as vacuolated hepatocytes, cell necrosis, pycnotic nuclei, cytoplasmic degeneration and necrosis leading to disintegration of hepatocytes. Gill of the treated fishes were showing pathological features like hyperplasia, lifting of secondary epithelium, squamous metaplasia, fusion of secondary lamellae, breakdown of pillar system and hyperaemia of cells.

Histology yields basic information on tissue disorders related to the general state of the animal. In the present investigation, the observed severe pathological changes in the liver, gills and kidney tissues obviously reflect a poor heath condition of fishes induced by prolonged exposure of lead and cadmium. Furthermore, it confirms liver, gills and kidney as the target tissue for toxicity of xenobiotics. It is concluded that though this type of histopathological analysis requires sacrificing of the target animal, is time consuming, and requires great expertise in tissue sectioning and interpretation, standard histology provides useful and reliable information on the health oh fishes.

Such tissue level alterations can induce the nutritional value of fish as an important edible commodity which in turn might negatively affect its market demand. It is concluded that the findings of the present histological investigations reveal a direct relationship between heavy metal exposure and histopathological disorders observed in several tissues. The current study reinforces the relevance of histopathology as a potent tool for monitoring contamination in aquatic environment.

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