AFLATOXICOSIS AND ITS AMELIORATION IN BLACK TIGER SHRIMP, *PENAEUS MONODON* FABRICIUS

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CERTIFICATE

This is to certify that the thesis entitled **"Aflatoxicosis and its amelioration in black tiger shrimp, *Penaeus monodon* Fabricius"** is an authentic record of research work carried out by Radhika Gopinath (Reg.No. 2236) under my guidance and supervision in Central Marine Fisheries Research Institute, in partial fulfilment of the requirements for 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 and associateship in any university.

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PREFACE

The spectacular progress scaled by aquaculture of penaeid shrimps has recently been put at risk by the dark clouds of disease manifestations. The etiological agents of many shrimp diseases in culture operations remain unknown. Diseases in shrimps can be caused by viral, bacterial or parasitic infestation, and also by other factors related to culture environment and feed. One such feed associated disease is established to be of mycotoxin origin caused by fungal contamination of improperly stored feeds.

Aflatoxin, a toxic contaminant produced by toxigenic fungi of the genus *Aspergillus,* during the processing and storage of feeds and feed ingredients, can cause abnormalities such as poor growth, physiological imbalances and histological changes that result in yield reduction and profitability of shrimp culture. In humid tropical and semi-tropical environments, the stored feeds in shrimp culture facilities are vulnerable to production of aflatoxins due to the prevalance of favourable conditions for fungal growth.

Very limited work on the potentiating factors of aflatoxicosis has been carried out. In the aquatic system, the animals are exposed to a wide variety of pathogens, pollutants, pesticides and stress factors. A diseased condition in an animal is the end result of not only one but the culmination of many factors. Hence the synergistic effects of two or many etiological agents can bring out toxicity in an animal.

A colossal challenge presently is the detoxification of aflatoxin contaminated foods and feeds as they can cause severe liver abnormalities in the consumers. Hence, aflatoxin and major toxic metabolites enjoy considerable importance due to their effect on human and animals health.

The present work is an attempt to elucidate the nutritional and pathological changes associated with aflatoxicosis in *P. monodon*, and to

determine the efficacy of vitamins E and K and an herbal powder, 'Amrita Bindu' in ameliorating the toxicity of aflatoxin B_1.

The thesis is organized into five chapters with a **General Introduction** and **Objectives** of the study in Chapter I. Chapter II deals with **Review of Literature** with special reference to aflatoxicoxis in land animals, fishes and shrimps. In Chapter III, the **Materials and Methods** of the study are given in detail. The **Results** are presented in Chapter IV and the **Discussion** in Chapter V. The **Summary** of the important findings and **Conclusions** follow the five chapters. The literature cited in the thesis is listed in the **Reference** section.

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List of Abbreviations

AB- Amrita Bindu

AFB_1- Aflatoxin B_1

ACP – Acid phosphatase

ALP – Alkaline phosphatase

ALT- Alanine transaminase

AST- Aspartate transaminase

LDH- Lacate dehydrogenase

ppb - parts per billion (μg/kg)

ppm - parts per million (μg/g or mg/kg)

Chapter I

Introduction

INTRODUCTION

The success tales of the developed and developing countries have revealed the fact that the natural resources, with diversified flora and fauna, are of paramount importance to the all round development of a nation. In this era of globalization, animal based food production systems offer bright scope for augmenting protein rich nutritious food for the future. Aquaculture represents one of the fastest growing food producing sectors in the world and is growing more rapidly than all other animal food producing sectors.

Aquaculture has made an impressive mark in global fish production in this age of dwindling marine resources and diminishing returns. Its contribution to the global supply of fish, crustaceans and molluscs increased from 3.9 percent of total production by weight in 1970 to 29 percent in 2001 according to FAO's State of World Fisheries and Aquaculture 2004 report (SOFIA). Aquaculture production including aquatic plants, reached 45.7 million tonnes by weight and $ 56.5 billion by value in 2000 (FAO, 2004).

Worldwide, aquaculture production has increased at an average compounded rate of 9.2 percent per year since 1970, compared with only 1.4 percent for capture fisheries and 2.8 percent for terrestrial farmed meat production systems (FAO, 2004). In 2003, global fish production was estimated as 132.2 million tonnes. More than 1 billion people worldwide rely on fish as an important source of animal proteins. The human consumption of fish is about 103 million tonnes and per capita utilization is about 16.3 kg. About 56% of the world's population derives at least 20% of its animal protein intake from fish, while small island states depend on fish almost exclusively (FAO, 2004).

To meet the increased demand for fish and to compensate for the extra buck spent on land and labour, aquafarmers are forced to intensify farming systems with high stocking densities. Such intensive and semi-intensive farming systems of fish and shrimp require nutritionally adequate, economically viable, optimally processed and eco-friendly feeds. About 50 to 60% of the total operational cost is spent on feeds because good quality feed promotes faster growth in fish. The total estimated industrial animal feed production in 2003 was 612 million tonnes, of which aquaculture feed contributed about 17.78 million tonnes (3%) and estimated to be 19.8 million tonnes in 2005 (FAO, 2004)

Feeds if not properly stored are ideal substrates for microbial contamination. The chance of fungal contamination is more in tropical countries like India, where high levels of humidity and temperature promote fungal infestation. Fungal infestation of feeds affects the shelf-life leading to substantial economic losses. Inappropriate means of bagging, storage and transport facilitate further fungal growth and ultimately toxin production.

Most of the aquaculture feeds and their ingredients have good nutritional profile to support growth of a wide range of toxigenic fungi. In the case of oil cakes, the residual oil becomes an important factor affecting the rate of toxic metabolite elaboration. In the tropics many farmers' transport and store feeds in substandard conditions. This can lead to the feed becoming moldy and contaminated with mycotoxins. Mycotoxins are produced as secondary metabolites by moulds on agricultural products before or after harvest and also during transport and storage. Mycotoxin contamination of food and feeds is a recurrent phenomenon, but attitude towards it has been far from consistent. As Goldblatt (1970) has pointed out that mycotoxin constitutes serious and ever present environmental health

hazard. It cannot be eliminated from our food supply. We must, as such, learn to live with them (Verma, 2001).

Among the mycotoxins, aflatoxins are the most toxic and have considerable interest in agriculture, livestock and aquaculture. Aflatoxins are extremely biologically active secondary metabolites produced by the fungi, *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are receiving increasing attention from researchers, the food industry, and the general public - firstly because they reduce production and secondly they remain as residues in animal tissues, which in turn affects the human metabolic system on being consumed. Aflatoxins are particularly important in aquaculture since their presence exerts a negative economic impact on relevant commerce as well as severe health problems after exposure to contaminated food and feed.

The discovery of aflatoxin in 1961 inspired a vast amount of research and focused attention on wider effects of aflatoxin. Aflatoxins are both acutely and chronically toxic to animals including man. They produce four distinct effects –acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Ueno and Ueno, 1978; Pier, 1981). Aflatoxin B_1 (AFB_1) is the most potent among naturally occurring carcinogens and is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC). Exposure to the most potent mycotoxin AFB_1 has also been suggested to increase Primary Hepatocellular Carcinoma (PHCC) risk (IARC, 1987).

No region of the world escapes the problem of mycotoxin. According to the United Nations Food and Agriculture Organization approximately 25% of the world's grain supply are contaminated and this equates to a

direct cost of billions of dollars due to the loss of crops and animals plus the hidden indirect costs incurred in monitoring the levels of aflatoxins in the crops and the decreased performance of farm animals that ingest aflatoxins and other mycotoxins (FAO, 2002). The moulds that produce mycotoxins tend to grow under warm, moist conditions, the same conditions that predominate in the tropics where most aquaculture is practiced. Crops raised during very dry or drought conditions or harvested during wet conditions, particularly with inadequately maintained equipment, are more prone to mycotoxin contamination (Anon, 1989).

One of the fastest growing aquacultural production sectors is that of penaeid shrimp. The share of farmed shrimp increased more than six fold from 1984 to 1999, contributing 1.1 million tonnes to the total penaeid production of 2.4 million tonnes in 1999 (FAO, 2002). World shrimp production is dominated by *P. monodon* that accounted for more than 50% of the production in 1999 (FAO, 2002). The culture of *Penaeus monodon* is constantly hampered by outbreaks of bacterial, viral, parasitic diseases and also by environmental and nutrition related diseases. One such constraint is the disease caused by fungal contamination of feed that often invites secondary infections.

Experimental studies of aflatoxicosis in shrimps were restricted to *P. vannamei* (Lightner *et al.,* 1982), *P. stylirostris* (Wiseman *et al.,* 1982; Ostrowski-Meissner *et al.*, 1995) and *P. monodon* (Boonyaratpalin *et al.*, 2001), the most cultivated penaeid shrimp in India and elsewhere. Kalaimani *et al.* (1998) have reported the presence of aflatoxin in imported and indigenous shrimp feeds in the range of 10 and 130 ppb collected from shrimp farms in Andhra Pradesh in India.

However, there has not been any concerted attempt to study the aflatoxicosis in Indian shrimps. The information on biochemical changes, ultrastuctural alterations, and synergistic effect of aflatoxins and heavy metals on *P. monodon* fed aflatoxin incorporated feed is limited. Besides there is no published information on amelioration of Aflatoxin B_1 toxicity in *P. monodon*. Hence the present work was undertaken with the following objectives.

- To document the pathological and immunological changes in *P. monodon* fed with AFB_1 incorporated diets.
- To delineate the histological and ultrastructural changes and determine the presence of AFB_1 residue in the shrimp body.
- To evaluate the growth performances and feed efficiency in *P. monodon* postlarvae fed AFB_1 added diets.
- To assess the interactive effect of heavy metals like copper and cadmium at sub-lethal levels in *P. monodon* postlarvae fed AFB_1 added diets.
- To decipher the ameliorative action of Vitamins E & K and a spicy herbal mixture, Amrita Bindu on AFB_1 in *P. monodon* sub-adults.

Chapter II

Review of Literature

2. REVIEW OF LITERATURE

2.1. Aflatoxins: Effects, Types and Occurrence

2.1.1. Historical perspective

Aflatoxins are polycyclic unsaturated compounds with a coumarin molecule flanked on one side by a bisfuran moiety and on the other side by either a pentanone for B series or a six-membered lactone for G series (Coulombe, 1991). Aflatoxins are secondary metabolites produced by certain species of fungi of the genus *Aspergillus.* Intensification in mycotoxin research was the result of concurrent disease outbreaks in poultry and fish during the 1960's in diverse geographic locations. The most prominent development was the report of severe losses of turkey poultry in Britain (Blount, 1961). Since the etiological agent involved in the disease was not known, the disorder was named 'turkey disease.' Examination of the feed source showed that a common factor in disease outbreak was the utilization of a Brazilian peanut in the rations (Blount, 1961; Sargeant *et al.*, 1961).

Wolf and Jackson (1963) and Sinnhuber *et al.* (1965) have subsequently demonstrated an interesting parallel between the developments associated with the identification of the etiological agent involved in the turkey disease and that of an epizootic liver cancer in hatchery reared rainbow trout. The outbreak of trout hepatoma was associated with the ingestion of toxic factors in the cottonseed meal (Sinnhuber *et al.*, 1965).

The causative agent and the responsible fungal species were subsequently identified by several workers (Nesbitt *et al.*, 1962; Sargeant *et al.*, 1963). To date only three species of fungi have been reported to produce aflatoxins. They are *Aspergillus flavus, A. parasiticus* and *Pencillium tuberculum.* The toxins produced by moulds are broadly classified as nephrotoxins, hepatotoxins and neurotoxins depending on the hematological

effects and general digestive disorders they cause. Aflatoxin comes under the category of hepatotoxins and targets its activities mainly on liver (Spensley, 1963).

2.1.2. Types

Over 200 different mycotoxins have been identified to date from feed ingredient sources. Although 17 aflatoxins have been isolated, only four of them are well known and studied extensively from toxicological point of view (WHO, 1979). Being intensely fluorescent in ultraviolet the four are designated B_1, B_2, G_1, G_2 representing their blue and green fluorescence in UV light. Two other familiar aflatoxins, M_1 and M_2 are in fact metabolites of B_1 and B_2 and labelled so because of their presence in milk of animals previously exposed to B_1 and B_2 (Stoloff, 1976). The aflatoxins display potency of toxicity and carcinogenicity in the order of AFB_1> AFG_1 >AFB_2 > AFG_2 as illustrated by their LD_{50} values for day old ducklings (Wogan *et al.*, 1971).

2.1.3. Occurrence

Food products contaminated with aflatoxins include cereals (maize, sorghum, pearl millet, rice, barley, beans, wheat), oilseeds (groundnut, soyabean, sunflower, cotton), spices (chillies, black pepper, coriander, turmeric, ginger), tree nuts (almonds, pistachio, walnuts, coconut) cassava, sweet potato and milk (Allcroft and Carnaghan, 1963; Schuller *et al.*, 1967; Newberne and Butler, 1969). Aflatoxins are also found in fruits particularly apples, beer and wine resulting from the use of contaminated barley, cereals and grapes for production. Mycotoxins also enter the human food chain via meat or other animal products such as eggs, milk and cheese as a result of livestock eating contaminated feed (Sharma and Salunkhe, 1991).

2.1.4. Factors favouring aflatoxin production

The moulds grow and produce toxins under conducive conditions, which involve adequate substrate (carbohydrates), moisture in the substrate (=13%), relative humidity (=70%), adequate temperature and oxygen (Lovell, 1984). Fungal growth and aflatoxin contamination are the consequence of interactions among the fungus, the host and the environment (Anon, 1989).

Water stress, high temperature stress and insect damage of host plant are the other factors, which favour mould infestation and toxin production. Specific crop growth stages, poor fertility, high crop densities, weed competition have been associated with increased mould growth and toxin production (Verma, 2001).

2.1.5. Effects of Aflatoxin on land animals

Aflatoxicosis has been studied in numerous animals including swine, cattle, goats, dogs, chickens, turkeys and laboratory animals (Miller *et al.*, 1984; Dalvi, 1986). Patterson and Allcroft (1970) divided animal species into two groups (a) susceptible- calves, chicks, ducklings, guinea pigs and pigs; and (b) relatively resistant- goats, sheep, rats and mice. Ducklings were found to be the best model for the bioassay of aflatoxicosis. Signs of acute aflatoxicosis in ducklings were similar to those in chicks and turkey poults and included anorexia, poor growth rate, ataxia and death (Carnaghan, 1965).

The aflatoxin pathway is similar to any other toxin; the aflatoxin ingested through the contaminated food accumulates in the blood and organs. The bioaccumulated mass of the toxin at lethal levels leads to death of the animal whereas at sublethel levels it leads to immunotoxicity,

genotoxicity, carcinogenicity, teratogenicity and other functional effects. Susceptibility of animals to toxic effects of aflatoxin varies with several factors such as breed, strain, age, nutritional status, amount of toxin intake and also the capacity of liver microsomal enzymes to detoxify AFB_1 (Veltman, 1984).

2.1.5.1. Toxicity of aflatoxin

In cattle, feeding aflatoxin at a level of 2 mg/kg showed liver lesions after 4 weeks of treatment (Allcroft and Lewis, 1963). AFB_1 in chickens have been reported to cause liver damage, decreased haemoglobin and hypoproteinemia (Brown and Abrams, 1965), liver lesions (Carnaghan *et al.,* 1966), decrease in weight gain and feed efficiency (Dalvi and McGowan, 1984). Butler (1964) recorded haemorhages in many organs, particularly in congested lungs and necrosis in myocardium, kidney and spleen in rats. Rogers *et al.* (1971) observed fatty livers, periportal hepatic necrosis and proliferation of bile ducts and surrounding connective tissue in male rats given LD_{50} of AFB_1. Madhavan *et al.* (1965) have recorded hepatotoxicty and lesions like fatty infiltration, biliary proliferation and portal fibrosis in two rhesus monkeys fed aflatoxins for 34 days until their death.

The clinical abnormalities and histologic lesions of aflatoxicosis were patchy necrosis in kidneys, pancreas and spleen of guinea pigs (Butler, 1966), centrolobular necrosis and fibrosis of liver in pigs (Krogh *et al.*, 1973), liver necrosis, shrunken hepatic cells with pyknotic nuclei and fatty change of hepatocytes in ducks (Newberne *et al.*, 1964), anorexia, icterus, weight loss; increased serum activities of liver specific enzymes and hepatic fibrosis and degeneration in goats (Miller *et al.,* 1984) and degeneration of hepatic cells, fibrosis and hyperplasia in rabbits (Krishna *et al.*, 1991).

2.1.5.2. Carcinogenecity

Aflatoxins are highly carcinogenic to some species such as rat (Wogan and Newberne, 1967) while they are acutely toxic to other species such as chicks (Forgacs and Carll, 1962). Butler and Barnes (1964) observed that concentration of aflatoxins in the range of 0.07- 4.0 ppm could induce liver tumors in rats. Liver carcinomas have also been reported in ducklings (Carnaghan *et al.,* 1965), rhesus monkeys (Adamson *et al.,* 1973) and rats (Newberne and Rogers, 1973)

2.1.5.3. Haematopoietic system

The effect of aflatoxin on the blood features of guinea pigs and albino rats revealed a depression in the total red blood cell and white blood cell count and prolonged blood-clotting time (Panda *et al.*,1975). Clark *et al.* (1986) reported that aflatoxin in the range of 0.05 to 0.4 mg/kg for 23 days in white rabbits produced marked reductions in the plasma activity of several blood coagulation factors.

2.1.5.4. Immunosuppression

Aflatoxin has been reported to cause hypoproteinemia and low globulin levels in ducklings (Brown and Abrams, 1965); reduction in the response of T-lymphocytes and failure to develop immunity following vaccination in turkeys and in chickens (Pier *et al.*, 1972); decreased serum immunoglobulin G levels, chemotaxis and poor phagocytic activity by heterophils and monocytes in chickens (Tung *et al.,* 1975; Chang and Hamilton, 1979). Reddy *et al.* (1983) reported a dose and time related response of immunological functions in mice fed aflatoxin incorporated diets. In guinea pigs that received aflatoxin at the rate of 0.06mg/kg body weight for three weeks there was reduction in the number of T-lymphocytes (McLoughlin *et al.*, 1984). The cell-mediated immunity was suppressed by

aflatoxin B_1 in rats (Raisuddin *et al.,* 1993; Sharma, 1993) and goats (Anilkumar and Rajan, 1986).

2.1.5.5. Biochemical effects

Since aflatoxin is the most common and most potent of the aflatoxin group, it has been studied extensively for biochemical effects on various experimental animals. Clifford and Rees (1966) tried to tabulate the successive stages in biological activity of aflatoxin on the rat liver cell, each step being a consequence of the previous one. The stages included i) interaction of aflatoxin with DNA and inhibition of the polymerases resoponsible for DNA and RNA synthesis; ii) suppression of DNA synthesis; iii) reduction of RNA synthesis and inhibition of messenger RNA; iv) alterations of nuclear morphology, and v) reduction in protein synthesis. Young rhesus monkeys given 0.5 to 1.0 mg mixed aflatoxin daily had elevated serum content of hepatic enzymes, elevated bilirubin and depressed albumin at 2 to 4 weeks (Tulpule *et al.*, 1964). Effects of aflatoxin on DNA and RNA metabolism and protein synthesis have been clearly elucidated by several workers in rats and ducklings (Wragg *et al.*, 1967; Neal and Godoy, 1976). Metabolic alterations caused by aflatoxins in chickens result in elevated lipid levels (Tung *et al.,* 1972), disruptions in hepatic protein synthesis (Tung *et al.*, 1975), immunosuppression and decreased plasma amino acid concentrations (Voight *et al.,* 1980).

2.1.6. Effects of Aflatoxin B_1 on finfishes

The effect of aflatoxin has been studied in different species of fishes such as trout, salmon, channel cat fish, common carp and nile tilapia (Ashley *et al.*, 1964; Halver *et al.*, 1966; Sinnhuber and Wales, 1974; Jantrarotai *et al.,* 1990).

2.1.6.1. Carcinogenesis

High incidence of hepatic tumours in rainbow trout were reported by Ashley and Halver (1961), and Halver (1965). Aflatoxin was found to be extremely carcinogenic to trout. The presence of aflatoxin at the level of 0.01 ppb in feed could produce neoplasm in trout (Halver *et al.*, 1966; Halver *et al.*, 1969; Ashley,1970). Embryonated eggs bathed in aflatoxin containing water at 1 ppm for 15 min to 1 hr produced hepatoma in 60-70 % of trouts hatched out of these eggs (Wales, 1979). Aflatoxin at high levels induced an acute toxin syndrome in trout with massive focal hepatic neurosis, branchial oedema and general haemorrhagic syndrome (Sinnhuber *et al.*, 1977). The presence of fish protein concentrate augmented tumourogenic activity of AFB_1 (Lee *et al.*, 1978).

The combination of rainbow trout and AFB_1 has become a model for xenobiotic impact due to trout's great sensitivity to this carcinogen. Trouts exposed to very low concentrations of this toxicant in feed have very high incidence of carcinogenesis (Sinnhuber *et al.*, 1978). Baver *et al.* (1969) found the intraperitoneal LD_{50} dose of AFB_1, in rainbow trout as 0.81mg/kg body weight. Tumour occurrence in trout has also been reported by Ruiz-Perez (1984), Rasmussen *et al.* (1986) and Metcalfe *et al.* (1988).

Signs of severe aflatoxicosis in rainbow trout are liver damage, pale gills, reduced erythrocyte concentration (Ashley, 1970), and necrosis, fibrosis and ductular proliferations in advanced tumours (Sinnhubur *et al.*,1968). Liver neoplasms, necrosis of hepatocytes and degenerative changes in the pancreatic tissue were observed in rainbow trout due to prolonged feeding of aflatoxin at a level of 0.4 mg/kg in the diet (Halver, 1969).

Wunder (1974) reported on the occurrence of giant cysts of 11 cm diameter in the liver of female *Salmo gairdneri* spawners of 3 kg weight fed on aflatoxin contaminated diet for 4 years. Kumura *et al.* (1976) suggested

aflatoxin as the aetiological agent for the occurrence of adenomatous polyps in the stomach of hatchery grown trouts.

Nakatsuru *et al.* (1990) observed a high rate of AFB_1 – DNA adducts formed in rainbow trouts when compared to coho salmon and suggested that the adduct formation could be taken as a dosimeter for estimating the degree of sensitivity of the fishes to aflatoxins. Nunez *et al.* (1990) studied the AFB_1 metabolism and toxicity in rainbow trout fry and found that histopathological lesions and DNA binding showed a linear dose response suggesting that cytotoxicity and carcinogenecity depended on aflatoxin conversion to electrophilic 8,9 epoxide.

Electron microscopy of classical trabecular hepatoma in rainbow trout was reported by Scarpelli *et al.* (1963) and Scarpelli (1967). The observed ultrastructural features were highly developed endoplasmic reticulum, absence of glycogen within the neoplastic cell, well-developed golgi complex with lamellae, vesicles and dense granules, large nuclei and nucleoli, dilated rough endoplasmic reticulum, poorly developed microvilli, increased number and size of intercellular spaces. Electrophoretic patterns of serum from normal and tumour bearing trout showed an increase in plasma components in hepatomatous fish (Snieszko *et al.,* 1966). Nunez *et al.* (1991) also carried out electron microscopic studies of aflatoxin B_1 induced hepatocellular neoplasms in rainbow trout and observed severe changes in all organs but more pronounced alterations were observed in liver and kidney.

Bailey *et al.* (1988) found that trout and salmon showed variations in sensitivity to carcinogenic effect of aflatoxins. Trout embryos exposed to 0.5 ppm AFB_1 for 15 min showed 62% tumour incidence 12 months later, while coho salmon under the same condition showed only a 9% incidence. Bailey *et al.* (1994) investigated the relative carcinogenecities of aflatoxin

B_1 and aflatoxicol in rainbow trout and observed that both produced the same phenotypic response and hepatocellular carcinoma.

Tilapia was found to be highly sensitive to AFB_1. Aflatoxin at seven different doses ranging from 0.94 to 3 mg/kg of feed for 25 days resulted in reduced growth rate, feed intake; and the liver damages were fatty infiltration of hepatocyte, nuclear and cellular hypertrophy, nuclear atrophy, cellular infiltration and necrosis (Haller and Roberts, 1980). In Tilapia, carcinogencity was not confined to the liver but produced wide range of neoplasms like renal tubular carcinoma, lymphoma and hepatoma. It also produced high mortality, lipoid degeneration and focal necrosis, reduction in serum protein levels, extensive necrosis of spleen and kidney parenchyma. The effects of dietary aflatoxin in Nile Tilapia (*Oreochromis niloticus*) were fatty degeneration, necrosis and fibroblast in liver (Chavez *et al.,* 1994).

Zhang-Quan *et al.* (1992) observed an increase in tumour incidence with higher temperature in rainbow trout. Curtis *et al.* (1995) have observed the influence of temperature on tumour incidence in rainbow trout. Fishes acclimatized to cool, intermediate and warm temperatures were exposed to 0.08- 0.12 ppm aflatoxin for 30 minutes. When the cool and warm temperature acclimatized fishes were reared at intermediate temperature after toxin exposure, tumour incidence showed a dramatic increase in the cool temperature group, while a drastic decrease in the warm temperature group. Thorgaard *et al.* (1999) reported a lesser incidence of induced tumours in triploid trouts than diploid trouts.

2.1.6.2. Effects on vital organs

Aflatoxins also bring about severe effects on vital organs like liver, kidney, thymus, spleen, and intestine. Svobodova and Piskac (1980) reported that in carps, aflatoxins did not produce any liver lesions, but higher doses like 20 and 200 ppm in feed caused histopathological

alterations like dystrophy of liver. Svobodova *et al.* (1981) and Nunez *et al.* (1990) have reported liver damage due to aflatoxins in rainbow trout fry characterized by swelling of hepatocytes and necrosis. Liver of channel catfish fed aflatoxin contaminated feed elicited marked variations from normal, which included necrotic foci with basophilic hepatocytes (Jantrarotai and Lovell, 1990). Acute toxicity of AFB_1 in channel catfish at a dose of 12 mg/kg body weight resulted in pale gills, kidneys, spleen, stomach and intestine of moribund fishes (Jantrarotai, 1991).

Jantrarotai (1991) studied the effect of aflatoxin in channel catfish, *Ictalurus punctatus* and observed necrosis and basophilia of hepatocytes. Chavez *et al.* (1994) reported severe damage to the liver of tilapia fed seven different levels of aflatoxins. Changes in the liver were fatty infiltration of hepatocytes, nuclear and cellular hypertrophy, nuclear atrophy, cellular infiltration, cellular basophilia and necrosis. Sahoo *et al.* (2001) reported necrotic and vascular changes in the liver of rohu (*Labeo rohita*) by acute toxicity of aflatoxin. Preneoplastic lesions in liver were observed as a major histopathological alteration during subchronic exposure. Anh Tuan *et al.* (2002) investigated the response of Nile tilapia to diets containing 0- 100 mg/kg AFB_1 for 8 weeks and reported that livers of fish fed diets containing 10 mg/kg toxin had excess lipofuscin and irregularly sized hepatocellular nuclei.

2.1.6.3. Immunosuppression

Arkoosh and Kaattari (1987) have observed reduced B cell memory in rainbow trout embryo exposed to AFB_1. Ottinger and Kaattari (1998) have reported the sensitivity of rainbow trout leucocytes to AFB_1 and found a decrease in lymphocyte proliferation and immunoglobulin production in response to mitogen lipopolysaccharide. Aflatoxin treated rohu, *Labeo rohita,* had reduced total protein and globulin levels, serum bactericidal

activities and bacterial agglutination titre when compared with the control group (Sahoo and Mukherjee, 1999).

2.1.6.4. Mutagenecity

Injection of 400 µg/kg of aflatoxin in *Salmo gairdneri* resulted in quantitative changes in the protein / DNA ratio of liver chromatin (Childs *et al.*, 1972). Al-sabti (1985) noticed that aflatoxin induced chromosomal aberrations in the kidney cells of cyprinids within 48 hours of injection. Krishna and Gupta (2001) showed that sub-lethal doses of aflatoxin produce fragmented, accentric and ring chromosomes in rohu and catla.

2.1.6.5. Haematopoietic system

Parashari and Saxena (1983) studied the toxicity of AFB_1 in the catfish *Clarias batrachus* and noticed leukaemogenic effect on blood leucocytes. Plumb *et al.* (1986) reported severe anaemia, low haematocrit values and mortality in channel catfish due to aflatoxicosis and aflatoxin at 10 ppm level in feed was highly effective in altering haematocrit values (Jantrarotai and Lovell, 1990). Acute toxicity in channel catfish resulted in sharp reduction in haematocrits, haemoglobin concentration and erythrocyte counts, whereas subacute toxicity caused anemia and liver necrosis in channel cat fish (Jantrarotai,1991). In Indian carp, *Labeo rohita*, blood failed to clot and levels of total serum protein, albumin and total leucocyte count were depressed when a diet containing 0.4 mg/kg AFB_1 were fed (Ottinger and Kattari, 1998).

2.1.6.6. Biochemical effects

Taylor *et al.* (1973) observed changes in ativity of liver enzymes like glucose-6-phosphate dehydrogenase, NADP-linked isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogense in rainbow

trout fed 20 ppb AFB_1. Juvenile rohu (200-300 g) fed 400 ppb aflatoxin B_1 showed changes in serum protein as evidenced by fall in total serum protein, albumin values and significant variation in globulin values and albumin / globulin ratio (George, 1998).

2.1.6.7. Aflatoxin residues in muscle tissue

Liver and kidney are considered to be the target organs for accumulating the toxin. Svobodova and Piskac (1980) and Svobodova *et al.* (1981) have not observed any accumulation of aflatoxin in fish muscles. Plakas *et al.* (1991) opined that there exists very low potential for aflatoxin accumulation in the edible flesh of channel catfish by consuming toxin contaminated feed. Ngethe *et al.* (1992) investigated by autoradiography and scintillation activity the fate of tritiated aflatoxin administered both orally and intravenously in rainbow trout for a period of 8 days and found the highest tissue concentrations in the liver followed by bile, kidney, pyloric caeca, eye and olfactory rosette. Horseberg *et al.* (1994) have reported on the deposition of tritiated aflatoxin in the head kidney and trunk kidney of rainbow trout and Nile tilapia following oral and intravascular administration and showed that hepatic accumulation of aflatoxin was more in rainbow trout than in nile tilapia revealing variations among species in accumulation of toxin. Wu (1999) studied the retention of diet related aflatoxin in the flesh and other tissues of channel catfish and found that toxic residues present in the flesh were proportional to the doses of toxin consumed.

2.1.7. Effects of AFB_1 in crustaceans

Only a few species of crustaceans have been tested for sensitivity to aflatoxin. *Artemia salina*, the brine shrimp, was found to have a 24 hr LC_{50} of 1.3µg /ml (Harwig and Scott, 1971). The copepod, *Cyclops fuscus* was

found to have 24 hr LC_{50} of approximately 1µg /ml aflatoxin B_1 (Reiss, 1972 a) and the water flea, *Daphnia pulex*, suffered 80% mortality in a solution of 1µg /ml aflatoxin B_1 in 24 hr (Sinnhuber and Wales, 1978).

Aflatoxins, produced by *A. flavus* and *A. parasiticus* may be a cause of disease in shrimp culture because culture facilities are typically located in humid tropical or semitropical environments, providing conditions favourable for the growth of *Aspergillus* spp and the production of aflatoxin in stored feeds.

Red discoloration or red disease was first noted in *Penaeus monodon* cultured in Taiwan by Liao (1977) which indicated that the development of red disease was subacute or chronic, with no evidence of an infectious aetiology, and suggested a link between feeding rancid fish and red disease, because the disease was not observed when care was taken to ensure that only fresh fish was fed. The disease has also been observed in captive wild adult *P.monodon* and in juvenile and adult cultured *P. monodon* in the Philippines and in pond reared *P.stylirostris* in Hawai (Liao, 1977). The principal lesion type observed were marked atrophy and necrosis of the hepatopancreas accompanied by an intense cellular inflammatory response. The aetiology of red disease being unknown, but because of the similarity of the hepatopancreatic lesions in red disease to those observed in aflatoxicosis, mycotoxins present in rancid or spoiled feeds or in the detritus of organically rich ponds were suggested as its cause (Lightner and Redman, 1985)

Studies at SEAFDEC, Philippines, showed that shrimps fed with diet containing aflatoxin (150 and 200 µg/g of feed) showed high incidence of reddening. Early signs of abnormalities observed were change of colouration of the pleopods from normal to reddish orange and reddening of the faecal matter. Histological observations showed severe damage to the hepatopancreas (Cruz and Tendencia, 1989). Jayasree *et al.* (2001) reported

mass mortalities of *P.monodon* in culture ponds of Andhra Pradesh, India, due to red disease characterized by red colouration of the body, presence of encrustations of fungal hyphae on carapace, appendages and gills, cessation in feed intake. Usage of locally made feed contaminated by *Aspergillus flavus*, the low salinity conditions in the culture ponds and the lack of water exchange were suggested as factors responsible for the disease outbreak.

The acute and sub-acute toxicity of AFB_1 to the marine shrimp *P. stylirostris* and *P.vannamei* were investigated. *Penaeus stylirostris* of 3 g average weight were exposed to a range of aflatoxin concentrations by intramuscular injection (2-160 μg AFB_1 /g body wt) and *P.vannamei* of 0.5 g average weight were fed different doses of aflatoxin (53-300 μg AFB_1/g feed). The histopathological alterations of aflatoxicosis in the aflatoxin-exposed animals were found to be time and dose dependent in the hepatopancreas, mandibular organ and in the haematopoietic organs (Lightner *et al.*, 1982).

. A marked intertubular haemocyic inflammation followed by encapsulation and fibrosis of affected tubules was observed in subacute aflatoxicosis. Other organs and tissues affected by aflatoxin were gills, heart, nerve cord and haematopoietic organs. Penaeid shrimps are relatively resistant to aflatoxin. The smallest dosage as 2 ppm of aflatoxin administered resulted in just detectable lesion development in the hepatopancreas. The 24hr LD_{50} was found to be from 90 to 200 ppm, and the single dose LD_{50} was found to be approximately 25 ppm (Lightner *et al.*, 1982).

Wiseman *et al.* (1982) studied the toxicity of AFB_1 in *P. stylirostris* by intramuscular injection and foud that the 24h and 96h LD_{50} for *Penaeus stylirostris* were 100.5 (78.3 to 129.0) and 49.5 (29.8 to 82.3) mg/kg respectively. Juvenile *P. vannamei* fed 50 to 300 ppm aflatoxin died within

4 weeks and showed lesions in the hepatopancreas, mandibular organ and haematopoietic organs (Wiseman *et al.*, 1982).

Lavilla-Pitogo *et al.* (1994) observed histopathological changes in *P.monodon* juveniles fed aflatoxin B_1 contaminated diets (26.5 to 202.8 µg/kg AFB_1 for 60 days). Shrimp fed diets with more than 50 ppb AFB_1exhibited haemolytic infiltration and fibrosis in the intertubular sinuses of the hepatopancreas and stated that the occurrence of more severe lesions in shrimp given higher doses of AFB_1, correlated with poor growth.

Growth and survival results from two indoor trials demonstrated that 3 weeks of exposure of juveniles of *P.vannamei* to 15 ppm and 3 ppm aflatoxin caused lethal and sublethal effects and that all the shrimps fed 15 ppm toxin diet died within 14 days and 3 ppm feed was not normally taken by shrimps. The FCR varied directly with AFB_1 levels from 50 ppb to 15 ppm and growth rate showed inverse relation with toxin levels. *P.vannamei* juveniles dosed with 400 ppb aflatoxin for 8 weeks showed a 17% reduction in final weight, 9% reduction in digestibility coefficient and a 23% increase in FCR relative to the controls (Ostrowski-Meissner *et al.*, 1995).

Boonyaratpalin *et al.* (2000) described the growth performance, blood components, immune function and histopathological changes in the black tiger shrimp, *P. monodon* of size 1.17 g average weight fed with different doses of AFB_1 (0 to 220 ppb) for 8 weeks. The total haemocyte counts, phenoloxidase activity as well as SGOT, SGPT in plasma showed increasing trends with increasing concentration of toxin. At concentrations of 74 ppb and above, atrophic changes of hepatopancreatic tubules, hyperplasia and necrosis were observed.

Divakaran and Tacon (2000) observed the potential for transmission of aflatoxin B_1 to humans through consumption of shrimp contaminated with this toxin. The residue analysis of *P. vannamei* fed diets dosed with 300, 400 and 900 ppb aflatoxin B_1 for 3 weeks showed that AFB_1 was

below detection limit of 2 ppb in shrimp faeces, whole shrimp or tail muscle.

Boonyaratpalin *et al.* (2001) studied the changes in blood components, growth performance, immune function and histology in *P. monodon* juveniles (0.7g) and adults (10±2 g) given diets supplemented with 0, 50, 100, 500, 1000 and 2500 ppb Aflatoxin B_1 and observed highly negative correlation between AFB_1 levels and average weight, weight gain and survival. They also observed marked histological changes in the hepatopancreas of shrimps fed 100 to 2500 ppb AFB_1, characterized by degeneration, atrophy, necrosis, encapsulation of necrotic cells and infiltration of connective tissue into interstitial tissues. Aflatoxin residues were also detected in head, shell and muscle of shrimps from all the groups and ranged from 13 ppb in 50 ppb group to 0.1 ppb in 2500 ppb group after 4 weeks.

2.1.8. Safe levels of AFB_1

The USFDA has regulated the levels of AFB_1 in food commodities to be processed into foods and has established an action guideline of 20 ppb for total aflatoxin. The action level for AFM_1 in milk has been set at 0.5 ppb (FDA, 1989). The European Union maximum permitted levels of aflatoxins in animal feeds and foods and FDA guidelines are presented in tables 2.1 and 2.2.

Table 2.1. European Union Maximum permitted levels of aflatoxins in animal feed and foods (FAO, 2002)

Aflatoxin	Foods and feeds	Animals
12 ppb	Dried fruits and nuts	
5 ppb	Animal feedstuffs	Cattle and sheep
2 ppb	Animal feed stuffs	Poultry and swine
1 ppb	Animal feed stuffs	Piglets and chicks

Table 2. 2. FDA Guidelines on maximum levels of aflatoxin in feedstuff (FAO, 2002)

Aflatoxin	Animals
20 ppb	Dairy, immature pigs, poultry, animal feeds, fish and shrimp feeds
100 ppb	Breeding animals
200 ppb	Finishing swine
300 ppb	Beef cattle

2.1.8.Acute toxicity of AFB_1 to various animals

The toxicity levels of AFB1 in different animals are represented in the table 2.3.

Table 2. 3. Toxicity levels of AFB_1 to various animals

Groups	Animals	Oral LD_{50} levels (mg/kg)	Source
Mammals	Rats	7.2-17.9	Edds (1973)
	Mouse	9.0	Edds (1973)
	Rabbit	0.3	Patterson (1973)
	Guinea pig	1.4-2.0	Butler (1966)
	Hamster	10. 2	Wogan (1973)
	Monkeys	7.8	Shank *et al.*, (1972)
	Baboon	2.0	Peers and Linsell (1976)
Aves	Ducklings	0.36-0.73	Newberne and Butler (1971)
	Turkey Poult	0.5	Butler (1964)
	Rats	7.2 –8.0	Butler (1964)
Fishes	Rainbow trout	0.5	Wales(1970)
Crustaceans	Copepod	1.0^{lc}	Reiss(1972 a)
	Brine shrimp	14.0^{lc}	Reiss (1972 b)
	Penaeid shrimps (*P. stylirostris*)	100.5^{im}	Wiseman *et al,.*(1982)

lc = LC_{50} at 24 hr (mg/l) ; im = intramuscular injection

2.2. Effect of other toxins and heavy metals on AFB_1 toxicity

Aflatoxins are produced only at appropriate conditions of temperature, humidity, moisture and substrate. The secondary fungal metabolites produced are temperature dependent and the yield is affected by the concentrations of trace metals like Manganese, Iron and Zinc (Weinberg, 1977).

Huff and Doerr (1981) evaluated the combined effect of aflatoxin and ochratoxin A (2.5 μg/g aflatoxin + 2.0 μg/g ochratoxin A) in broiler chickens. Angsubhakorn *et al.* (1981) suggested that hepatocellular carcinomas developed in 79% of rats fed 25 ppm dimethylnitrosamine and 1 ppm AFB_1. The presence of dieldrin in the diet with 6 ppb aflatoxin B_1 increased the incidence of hepatocellular carcinomas in rainbow trout (Hendricks *et al.,* 1979). The Mt. Shasta rainbow trout (*Salmo gairdneri*) was found to produce hepatocellular carcinoma, when administered diets with both aflatoxicol and cyclopropenoid fatty acids (Schoenhard *et al.*, 1981).

Osuna and Edds (1982) studied the interaction of cadmium and aflatoxin B_1 on pig's performance and hematology for 5 weeks and found that all the pigs had developed severe anemia by the 4th week of the experiment. Morrissey *et al.* (1987) reported the combined effects of aflatoxin B_1 and cyclopiazonic acid on Sprague-Dawley rats and reported weight loss and gross pathological changes like icterus, shrunken liver and lesions in the kidney at the cortico-medullary junction.

The cyclopropionic fatty acids and gossypol present in cottonseed meal have been shown to serve as co-carcinogens with aflatoxin in rainbow trout. The individual and combined effects of feeding diets containing moniliformin (M) and aflatoxins in chicks were evaluated by Kubena *et al.* (1998) which revealed additive or less than additive toxicity, but not toxic

synergy, for most parameters when chicks were fed diets containing the combination of 100 mg M and 3.5 mg AFB_1/kg of diet.

Although there are studies demonstrating the individual toxicity of copper and cadmium to *P. monodon* (Guo and Liao,1992.; Chen and Lin, 2001; Sulaiman and Noor ,1996; Munshi *et al.,* 1997), there has not been any attempt to evaluate the synergistic effect of aflatoxins and heavy metals in aquatic animals including shrimps.

2.3. Detoxification of aflatoxins

The contamination of animal feed with mycotoxins is a problem faced by farmers worldwide. The contamination of diets by aflatoxins and the carry-over of the toxic residues through the food chain have to be accurately controlled (Ramos and Hernandez, 1997). The consumption of toxin-contaminated diet may induce acute and long-term chronic effects resulting in a teratogenic, carcinogenic (mainly for liver and kidney), oesterogenic or immunosuppressive impact not only on animals but also on man (Steyn and Stander, 1999)

In addition to the toxic effects, a mycotoxin contaminated diet may lead to other consequences like feed refusal, poor feed conversion, diminished body weight gain, increased disease susceptibility due to immune suppression, and interference with reproductive capacities which are responsible for great economic losses (Anon, 1989). Aflatoxin contamination of foods and feeds can result from fungal contamination before harvest as well as during harvesting and storage operations. Unquestionably prevention is the best method for controlling mycotoxin contamination (Park *et al.,* 1988). Should the contamination occur, however, the hazard associated with the toxin must be removed if the product is to be used for food or feed purposes (Sharma and Salunkhe, 1991).

2.3.1. Detoxification and amelioration methods

2.3.1.1. Physical, Chemical and biological methods

In order to prevent mycotoxicosis, several pre-harvest and post-harvest technologies and biological, chemical and physical methods have been tested (Doyle *et al.*, 1982; Ramos and Hernandez, 1997). An efficient amelioration agent should aim at the following factors: ability to bind a wide range of mycotoxins, low effective inclusion rate in feed, rapid and uniform dispersion in the feed during mixing, heat stability during pelleting, extrusion and during storage, no affinity for vitamins, mineral or other nutrients or additives, high stability over a wide pH range and bio-degradable after excretion (Park *et al* ., 1986).

Physical removal of discoloured, damaged or inadequately developed kernels is the decontamination technique most widely used by the peanut industry, but such procedures are not practical for corn or cottonseed (Ashworth *et al.*, 1968). Unfortunately, mycotoxins diffuse away from the mycelia, and products having no visible evidence of mold damage, can contain mycotoxins at significant levels, therefore physical removal may not effectively detoxify the material. Alternative decontamination procedures are necessary to address these situations. The approach taken by most researchers has been toward chemical inactivation of the toxin (Goldblatt and Dollear, 1977).

In the case of peanuts and Brazil nuts, those exhibiting a blue fluorescence under UV light, indicating possible presence of aflatoxins, can be mechanically or electronically sorted (Ashworth *et al.*, 1968). Since fungal infested nuts are often lighter than healthy ones, it is possible to remove the contaminated ones using pneumatic separation (Galblatt, 1970). The possibility of removal of toxins from contaminated grains, seeds and nuts has been considered from the legal as well as practical standpoint.

Extensive studies have been carried out on the use of ammonia to decontaminate aflatoxin contaminated feeds, primarily cottonseed, corn and peanut products. The average ammoniation costs vary between 5 and 20% of the value of the commodity (Coker, 1998). The ammonia treated product may be subsequently used for animal feed (Galblatt, 1970). In the mechanism of ammonia treatment on AFB_1, it was observed that the molecular structure of the toxin is irreversibly altered if exposure to ammonia lasts long and in contrast, if exposure is not sufficiently protracted, the molecule can revert to its original state. Animal feeding studies utilizing ammonia have been conducted in ducklings, turkeys and rats (Park *et al.*, 1988). Main drawbacks of this kind of chemical detoxification are the ineffectiveness against other mycotoxins and the possible deterioration of the animal's health by excessive residual ammonia in the feed. (Park, 1993). The disadvantages of ammonia treatment are mainly related to the need to build special plants as ammonia corrodes metal and becomes explosive in the air at mixtures over 15% in volume (Piva *et al.*, 1995).

Feed manufacturers have increasingly incorporated mould inhibitors in their diets or applied them to raw materials in storage. Most of the products used are low molecular weight organic acids and their salts such as propionic acid (Nahm, 1995). Salts of the acids tend to last longer but are not as effective as volatile free acids. Volatile free acids achieve better penetration but are dispersed more rapidly. These products are generally effective at inhibiting the growth of mould but do not have any effect on toxins already present in the feed or raw material (Goldblatt and Dollear, 1977) Extraction of mycotoxins such as aflatoxins is feasible, however most extraction procedures result in the removal of nutrients. Aqueous solutions of sodium bicarbonate or calcium chloride have been suggested. But this procedure removes a large part of the protein and essential minerals and vitamins (Sreenivasamurthy *et al.*, 1967).

Biological methods of decontamination include fermentation procedures with microorganism; one example is the conversion of Aflatoxin B_1 particularly by *Flavobacterium auranticum* to harmless degradation products, but the conversions however are generally slow and incomplete (Sweeney and Dobson, 1998). Verma *et al.* (2001) have reviewed the different detoxification methods of aflatoxin followed: some of them were heat treatment, gamma and UV radiation, exposure to sunlight, use of moderately polar solvents, certain microorganisms and aflatoxin degrading enzymes and these methods were able to destroy 50-80% aflatoxins.

2.3.2. Chemisorptions

Mineral clay products such as bentonites, zeolites and aluminosilicates have been found to be effective in binding/adsorbing mycotoxins. Among these, aluminosilicates have been found to be more effective (Ramos *et al.,* 1996). Hydrated sodium calcium aluminosilicate (HSCAS) can selectively combine with AFB_1. Inclusion of 0.5% HSCAS in rations has been shown to ameliorate the deleterious effect of 0.5 ppm aflatoxin in growth rate and mortality in a week old broiler chicken (Beaver *et al.,* 1990). HSCAS at 1.0% of the feed (10 kg per tonne) could significantly diminish the adverse effects of aflatoxin in chickens, pigs and cows (Scheidler, 1993). Oguz *et al.* (2000) studied the effect of clinoptilolite, a natural zeolite (2.5 mg/kg diet) in broiler chickens fed 2.5 mg /kg AFB_1 and evaluated the ability to reduce the deleterious effect of aflatoxins. Addition of HSCAS (5 g/kg) to aflatoxin contaminated diet in rats resulted in a significant improvement in the haematological and biochemical parameters, mineral retention and histological picture of both liver and kidneys (Abdel-Wahab *et al.,* 1999).

2.3.3. Natural antioxidants and chemopreventors

Eisele *et al.* (1983) observed the effects of antioxidants like butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), mono-tert-butylhydroquinone (TBHQ) and ethoxyquin (EQ) at a level of 5.56 mmol in 100 g oil/kg diet for 6 wk. on the hepatic mixed-function oxidase system of rainbow trout and suggested that dietary antioxidants could alter carcinogen activation and detoxification mechanisms in the hepatic microsomes of rainbow trout. Beta naphthoflavone (BNF) and indole 3-carbinol (I3C) have been observed to give protection against hepatocarcinogenecity and reduced AFB_1 binding to DNA in rainbow trout (Nixon *et al.*, 1984). Fukayama and Hsieh (1985) proved that butylated hydroxytoluene (BHT) pretreatment (0.5% in the diet for 10 days) protected male rats from the carcinogenic effects of AFB_1 by enhancing the detoxification and excretion of the mycotoxin.

Dietary supplement of chlorophyll inhibited AFB_1-DNA binding in trouts (Yun *et al.*, 1995; Dashwood *et al.*, 1998). The flavanoid, ternatin from *Egletus viscosa* was effective in combating AFB_1 induced toxicity, measured in terms of lipid peroxidation, oxidative DNA damage and histological studies to assess hepatocellular necrosis and bile-duct proliferation in rats (Souza *et al.*, 1999). Bhattacharya (1987) reported the modifying role of nineteen vitamins including some derivatives that have been tested for their ability to suppress mutagenic activity of aflatoxin B_1 towards *Salmonella typhimurium* strain TA100 activated with a rat-liver metabolic activation system.

Firozi *et al.* (1987) studied the effect of vitamin A and some derivatives on the formation of DNA adduct by aflatoxin B_1 in an *in vitro* reaction catalyzed by rat liver microsomes and reported that retinol, retinal, all trans retinoic acid and two retinyl esters were found to inhibit formation of AFB_1 adduct with microsomal protein in a dose-dependent manner.

Verma *et al.* (2001) enumerated the ameliorative role of Vitamin A on aflatoxin-induced cytotoxicity *in vitro*. Aflatoxin induced haemolysis was found to get reduced on addition of Vitamin A (125-1250 IU/ml) in the incubation medium. Webster *et al.* (1996) demonstrated that hepatocarcinogenesis induced by aflatoxin B_1 was more pronounced in rats maintained on a riboflavin-deficient diet compared to that on a normal diet and the increased damage was reversed on riboflavin supplementation.

Salem *et al.* (2001) evaluated the effects of ascorbic acid on productive and reproductive characteristics of mature male rabbits given two sublethal doses (15 or 30 µg /kg of body weight) of AFB_1 for 9 days and concluded that ascorbic acid alleviated the negative effects of AFB_1 in a dose-dependent manner. Yousef *et al.* (2003) evaluated the effectiveness of L-ascorbic acid (AA) in alleviating the toxicity of aflatoxin B_1 in male white rabbits fed 30 picograms AFB_1 plus 20 mg AA/kg BW. Sahoo and Mukherjee (2003) examined the immunomodulatory effect of high levels of dietary vitamin C in healthy and immunocompromised rohu (*Labeo rohita*) treated with aflatoxin B_1 (1.25 mg /kg body weight intraperitoneally). High dietary vitamin C, at 500 ppm for 60 days enhanced the non-specific immunity of fish and offered protection against *Aeromonas hydrophila* infection in both healthy and immunocompromised fish.

Sahoo and Mukherjee (2002) have reported the immunomodulatory effect of beta-1, 3 glucan, levamisole and vitamins C and E, in rohu (*Labeo rohita* Ham.), intraperitoneally injected with aflatoxin B_1 at 125 mg/ kg body weight .The results demonstrated that all the four immunomodulators were capable of significantly enhancing the specific immunity and reducing the mortality in immunocompromised fish. High levels of a-tocopherol (Vitamin E) raised the specific immunity, nonspecific resistance factors and disease resistance capacity when compared with AFB_1 exposed Indian major carp, *Labeo rohita* (Sahoo and Mukherjee, 2002). Karakilicik *et al.*

(2004) investigated the effects of vitamin C and E on liver enzymes and other biochemical parameters in rabbits experimentally exposed to AFB_1 (0.1 mg AFB/kg diet) and observed that vitamins C, E and C+E partially prevented an increase in the liver enzymes and some of the biochemical parameters induced by AFB_1.

Menadione sulphate (Vitamin K) has been reported for control of aflatoxicosis in channel cat fish (Cowey *et al.*, 1985). Vitamin K (5 mg/kg), phenylbutazone (50 mg/kg) and sulfamethoxine (50 mg/kg) were able to suppress the increase in whole blood clotting time caused by AFB_1 (25 µg/kg) in albino rats (Asuzu *et al.,* 1988).

2.3.4. Efficacy of Food additives, beverages, spices and herbs on AFB_1 toxicity

Food additives such as turmeric, and active ingredient curcumin, asafoetida, butylated hydroxyanisole, butylated hydroxytoluene and ellagic acid were found to inhibit the mutagenesis induced by aflatoxin B (0.5µg / plate) in *Salmonella* strains. Dietary administration of turmeric, garlic, curcumin and ellagic acid to rats significantly reduced the occurrence of hepatocelluar neoplasm (Soni *et al.,* 1997). Turmeric and asafoetida were also useful in reducing AFB_1 production in cultures of *Aspergillus parasiticus* (Soni *et al.*, 1992). Curcumin, present in turmeric reversed AFB_1 induced tumor production in mouse (Huang *et al.*, 1992). Yen and Chen (1996) observed that catechins (flavones), caffeine, phenolic compounds, ascorbic and lipid soluble components like tocopherols and carotenes present in tea could suppress partially the mutagenecity of AFB_1.

'Amrita Bindu' (AB) prepared by mixing five salts, three spices (ginger, pepper and long pepper) and the herbs *Cyperus rotundus* and *Plumbago zeylanic* is a brown powder with a spicy odour and taste. The salt spice herbal mixture has been found to provide protection against MNNG

(N-methyl.N'-nitro-N-nitroguandine,a carcinogenic nitrosamine) induced lipid peroxidation and resultant tissue degeneration in rats (Shanmugasundaram *et al.*, 1994). The antineoplastic nature of AB is attributed to its combined effect on providing antioxidant defences and the absorption of the nitrosamine on the insoluble portion of AB and its faecal elimination. The presence of AB in the diet controls the free radical and oxidant induced changes in the liver and brain cells and increases glutathione-S-transferase facilitating the removal of the aflatoxin from the system (Sujatha, 1990).

Madhusudhanan *et al.* (2001) have reported the protective effect of Amrita Bindhu against acute aflatoxin induced alterations of the antioxidant status in *Labeo rohita* of size 2.9 ± 0.3 g. *L. rohita* administered with 1:1 mixture of 20% Amrita Bindu and 100 ppb of aflatoxin B_1 in oil intraperitoneally for 10 days showed improved performance of the antioxidant and detoxification enzymes in muscles and liver.

2.3.5. Use of yeast and yeast extracts.

In broiler chicks, *Saccharomyces cerevisiae* (yeast) at 0.1% level restored the serum concentrations of total protein and albumin caused by 5 ppm aflatoxin (Stanley *et al.*, 1993); 1% inclusion reduced the severity of aflatoxicosis (Victor *et al.*, 1993) and 0.05% level showed moderate amelioration of aflatoxin toxicity on serum cholesterol and cellular immune response (Raju *et al.*, 2004). Raju and Devagowda (2000) reported the beneficial effect of esterified-glucomannan, a cell wall derivative of *Saccharomyces cerevisiae* (1 g/kg) on performance and organ morphology, serum biochemistry and haematology in broilers exposed to aflatoxins and combinations of mycotoxins.

2.3.6 Other chemicals and enzymes

Liu *et al.* (1998) reported the detoxification of aflatoxin B_1 by multienzyme isolated from mycelium pellets of *Armillariella tabescens*. Broilers treated with N-acetylcysteine (NAC) (800 mg/kg body weight) plus AFB_1 (3 mg/kg of feed) were shown to be partially protected against negative effects induced by AFB_1 on performance, liver and renal damage and biochemical alterations (Valdivia *et al.*, 2001). Citil (2005) demonstrated that L-carnitine brought about the inhibition of lipid peroxidation by enhancing antioxidant capacity in quails with chronic aflatoxicosis given both 60µg total aflatoxin/kg diet and 200 mg L-carnitine/litre in the drinking water for 60 days.

Most of the research on aflatoxicosis has been done in rats and finfishes especially rainbow trout considering their high susceptibility. There is much less information in crustaceans but there is convincing evidence that aflatoxins could be associated with reduced performance of a number of aquaculture species. In India, majority of the aquacultural farms are located in the coastal areas where high temperature and humidity prevails, hence there are more chances of fungal contamination of feeds. However, there has not been any comprehensive work on aflatoxicosis or its amelioration in Indian shrimps.

Chapter III

Materials and Methods

3. MATERIALS AND METHODS

Experiments were conducted in the wet laboratory of Central Marine Fisheries Research Institute (CMFRI), Kochi. The formulation and preparation of feeds and biochemical analyses of feeds and feed ingredients were carried out in the Nutrition laboratory of CMFRI. The analysis of haemolymph and processing of the shrimp tissue for histology were carried out in the Pathology laboratory at CMFRI. Processing of shrimp tissues for ultrastructural studies was carried out in the elecrtronmicroscope facility of CMFRI.

Four experiments were conducted to analyse the effect of aflatoxin B_1. The first experiment was to elucidate the effect of aflatoxin B_1 on growth performance in *Penaeus monodon* postlarvae. The second experiment was to evaluate the synergistic effect of aflatoxin B_1 and heavy metals, copper and cadmium in *P.monodon* juveniles. The pathological and immunological changes caused by different doses of aflatoxin B_1 in diets were studied in *P.monodon* sub-adults in the third experiment. The detoxification effect of herbal powder and vitamins E and K on aflatoxin B_1 was studied in *P.monodon* sub-adults in the fourth experiment.

3.1. Experimental animals

The postlarvae for the first and second sets of experiments were obtained from Cochin Aqua hatchery at Malipuram, Ernakulam District, Kerala. About 1000 numbers of *P.monodon* postlarvae (PL 10) were packed in oxygenated polythene bags of two-litre capacity with filtered seawater of 20 ± 2 ppt salinity and transported to the experimental facility of CMFRI. The polythene bags containing seeds were then placed in the FRP holding tanks of 500 l capacity for one hour and then released slowly into the tanks containing seawater of 20 ± 2 ppt salinity. The postlarvae were acclimatized

for a week. Before the initiation of the experiment, the postlarvae were evenly distributed into different treatment tubs.

For the third experiment, *P.monodon* sub-adults of size 7.5 ± 0.72 g were collected from Matsyafed shrimp farms, Vypeen, Ernakulam District, Kerala. The shrimps were collected using cast nets and hand nets in the morning hours between 0700 and 0900 hrs. The environmental parameters observed in the rearing ponds were salinity 16± 2 ppt, temperature 27 ± 2 °C and dissolved oxygen 5.6 ± .05 mg/l. About 250 shrimps were transferred to 25 l carbouys (20 numbers) filled with pond water and aerated with a battery operated aerator and transported in jeeps to experimental facility at CMFRI, Kochi. The shrimps were released into FRP tanks of 1-ton capacity using a small hand net.

For the fourth experiment, 120 numbers of *P.monodon* sub-adults of size 11.3 ± 1.24 g were obtained from an extensive shrimp farm at Matsyafed, Vypeen, Ernakulam District, Kerala. The shrimps were collected using cast nets and hand nets in the morning hours between 0700 and 0900 hrs. The environmental parameters recorded in the rearing ponds were salinity 15 ± 2 ppt, temperature 29 ± 1 °C and dissolved oxygen 5 ± 0.04 mg/l. The shrimps were brought to wet lab of CMFRI in aerated carbouys of 20 l capacity and transferred to FRP holding tanks of 1ton capacity for acclimatization.

3.2. Water quality parameters

Seawater of salinity 35 ppt., obtained from Kannamaly seacoast and transported to the wet lab by tanker lorry, was used for all the four experiments. The seawater was pumped into the storage tank of 10-ton (10000 l) capacity and allowed to settle down for two days. The seawater used for all the experiments were pre-chlorinated with liquid sodium

hypochlorite (200 ppm) and vigorously aerated for two days in one-ton tank exposed to sunlight.

Dissolved oxygen, salinity, pH, temperature, ammonia levels in the seawater were determined. Dissolved oxygen was determined by Winkler's method (Strickland and Parsons, 1972); salinity was determined using a refractometer (ERMA, Japan); pH was recorded using a digital pH meter and temperature monitored using a mercury thermometer. Genesys 10 UV/VIS Spectrophotometer (Thermospectronics, USA) was used for estimation of ammonia by phenol-hypochlorite method as per Strickland and Parsons (1972). Temperature was noted daily at 0900 hours; dissolved oxygen and pH were determined at three days interval and ammonia was estimated once a week.

3.3. Experimental diets

3.3.1. Preparation of stock solution and working solution of Aflatoxin B_1.

Pure crystalline powder of Aflatoxin B_1 was obtained from Sigma chemical company, St. Louis, MO, U.S.A (Product Name A6636). 50 mg of aflatoxin B_1 was dissolved in 5 ml of chloroform to form stock solution containing 10 mg AFB_1/ml of chloroform. From this, working solution was prepared by adding 1ml of the stock solution to 49 ml of chloroform (10 mg aflatoxin in 50 ml of chloroform). The working solution was stored in amber coloured bottles sealed tightly with teflon and cellotape and stored under refrigeration. Handling of the toxin was carried out in a closed chamber using a glove box. Before addition of the toxin into experimental feeds, the required amount of toxin dissolved in chloroform from the working solution was taken in a glass beaker, evaporated in a water bath and replaced with equal volumes of ethanol.

3.3.2. Processing of Feed Ingredients

The feed ingredients chosen for preparing the experimental diets were fish meal, shrimp meal, clam meal, soyabean meal as protein sources, wheat flour as carbohydrate source and codliver oil as lipid source. Dry fishes (anchovies), dry shrimp (*Metapenaeus* and *Penaeus* spp.) and fresh clams were purchased from the local market and pulverized in a hammer mill (<250μm) after oven drying for 24 hours at 50°C. The powdered feed ingredients were stored in tightly packed containers. Soyabean powder and wheat flour were obtained from local feed suppliers. Minerals and vitamins were procured from Nice Chemicals, Cochin.

3.3.3. Proximate Composition

Moisture, crude protein, crude fibre levels in the feed and feed ingredients were determined as per AOAC (1990). Crude lipid (CL) was extracted by soxhlet extraction with petroleum ether (BP 60-80°C), ash content was determined as the residue remaining after incineration of sample at 550°C in a muffle furnace for 12 hours. Crude protein (CP) estimation was carried out using the Kjelplus KPS-020 (Pelican, Bio-innovations. Pvt. Ltd.) semi-automatic system and the titration using the Titroline 96 (Schott) and Nitrogen Free Extractives (NFE) was estimated as the difference (NFE=100-(CP+CL+CF+ Ash +AIA) (Table 3.1).

Fig. 3.1. Flow chart for estimation of Dry matter

Weighed a clean and dry aluminium cup (W)

↓

Weighed approximately 5g sample in the cup (W_1)

↓

Kept in hot air oven at 100 °C for 2 hours

↓

Cooled in a desiccator and weighed (W_2)

$$\text{Dry matter (\%)} = \frac{W_2 - W}{W_1 - W} \times 100$$

Fig. 3.2. Flow chart for estimation of Crude ash

Weighed clean silica crucible (W)

↓

Weighed approximately 3 g sample with crucible (W_1)

↓

Kept in hot air oven at 55°C overnight and incinerated in a muffle furnace at 600°C

↓

Cooled to room temperature in a desiccator and weighed (W_2)

$$\text{Crude ash (\%)} = \frac{W_2 - W}{W_1} \times 100$$

Fig. 3.3. Flow chart for estimation of Crude protein

Weighed approximately 0.5 g of sample in a digestion tube

↓

Added 1 g digestion mixture (K_2SO_4 + $CuSO_4$ in 9:1)

↓

Added 5 ml of Conc. H_2SO_4 and digested in digester (Kjelplus) at 350°C

↓

Cooled to room temperature after solution became clear and made up to 100 ml

↓

Took 10 ml sample into distillation tube and added 10 ml 40% NaOH in a distiller (Kjelplus) and distilled for 6 min.

↓

Released ammonia was collected in 10 ml mixed indicator

↓

Resultant green solution was titrated against 0.1 N HCl using an autotitrator (Titroline 96)

$$\text{Crude protein (\%)} = \frac{\text{Vol. of acid (ml)} \times 0.0014 \times 100 \times 100 \times 6.25}{\text{Weight of sample (g)} \times 10}$$

Fig.3.4. Flow chart for estimation of Crude fat

Weighed 3 g sample into a Whatman paper thimble and plugged using absorbent cotton (W). Placed the thimble in a soxhlet extraction unit

↓

Weighed a round bottom flask (W_1)

↓

Assembled in soxhlet extraction unit

↓

Added petroleum ether (BP.40–60°C) to the soxhlet

↓

Started water circulation in the condenser and set hotplates on bench to 58–60 °C and continued extraction for 8 hours (app. 256 cycles)

↓

Removed the round bottom flask (RBF) from assembly and dried in hot air oven

↓

Cooled the round bottom flask in dessicator and weighed (W_2)

$$\text{Crude Fat} = \frac{W_2 - W_1}{W} \times 100$$

Fig. 3.5. Flow chart for estimation of Crude fibre

Weighed 2 g. fat-free sample into 1 litre Berzelious spoutless beaker (W)

↓

Added 200 ml of 1.25 % H_2SO_4, covered the mouth with RBF containing cold water and digested for 30 min.

↓

Filtered through 100µm bolting silk by applying suction and repeatedly washed with warm water

↓

Residue was washed with 200 ml 1.25% NaOH into the beaker for alkaline digestion

↓

Filtered the contents into gooch crucible with a final rinse using acetone. Oven dried the gooch crucible at 55°C overnight (W_1)

↓

Incinerated in muffle furnace at 600°C and cooled to room temperature in a desiccator and weighed (W_2)

$$\text{Crude fibre (\%)} = \frac{W_1 - W_2}{W} \times 100$$

Table 3.1. Proximate composition of feed ingredients

Ingredients	Moisture	Dry matter	Crude Protein	Ether extract	Ash	Crude fibre	‡ NFE
Fish meal	5.2 ± 0.05	94.8 ± 0.05	65.5 ± 0.31	4.2 ± 0.22	21.4 ± 0.07	3 ± 0.184	3 .7 ± 0.17
Shrimp meal	6.7 ± 0.18	93.3 ± 0.18	64 ± 0.37	3.03 ± 0.04	16.05 ± 0.4	4.6 ± 0.181	5.62 ± 0.07
Clam meal	11 ± 0.2	89 ± 0.2	56.7 ± 0.41	9 ± 0.26	5.2 ± 0.21	0.1 ± 0.162	28.9 ± 0.43
Soya bean meal	6.9 ± 0.38	93.1 ± 0.38	51.42 ± 0.34	0.64 ± 0.02	7 ± 0.45	2.8 ± 0.187	31.24 ± 0.1
Wheat flour	9.5 ± 0.02	90.5 ± 0.02	12.45 ± 0.38	1.59 ± 0.03	1.15 ± 0.12	1.1± 0.06	74.21± 0.26

‡ NFE = 100 – (Crude protein + Ether extract + Ash +Crude fibre)

3.3.4. Formulation and preparation of experimental diets

Feed formulation was carried out using Microsoft Excel software. Vitamin and mineral premix were prepared using a blender with cellulose as filler to achieve uniform distribution. Vitamin mix was prepared without incorporating fat-soluble vitamins and the hygroscopic choline chloride. Fat-soluble vitamins were mixed with fish oil before feed preparation. Choline chloride was dissolved in distilled water and mixed at the time of dough preparation.

The dry pulverized feed ingredients: fishmeal, shrimp meal, clam meal, soyabean meal and wheat flour were accurately weighed and mixed in a blender. The mixture was moistened with water containing the required amount of gelatin and steam cooked for 10 minutes. After cooling, cod liver oil, vitamins, minerals were mixed uniformly in a kneader. The dough was partitioned into different portions for the control and treatment feeds, and the working solution of aflatoxin B_1 at the required concentration was added to the feed and thoroughly mixed. A hand-pelleting machine with 2mm die was used to extrude the dough. The pellets were oven-dried at 60°C

overnight. After drying, the pellets were broken into small crumbs and stored in airtight containers. The prepared feeds were analyzed for proximate composition.

3.4. Survey of feeds and feed ingredients

According to FAO, the permissible levels of aflatoxin in animal feeds should be below 20 ppb (FDA, 1989). Kalaimani *et al.* (1998) have reported the presence of 10-130 ppb aflatoxin B_1 in indigenous and imported shrimp feeds from Andhra Pradesh. A survey was conducted to ascertain the presence of aflatoxin in different feed and feed ingredients from shrimp farms around Vypeen, Ernakulam District, Kerala. The feed and feed ingredients were analysed for the presence of AFB_1 residue using a fluorometer (VICAM V1 Series 4).

Procedure: Using aflatoxin calibration standards the fluorometer was calibrated. The aflatest developer solution and methanol: water (80: 20 by volume) was prepared fresh. The reagent blank (1 ml methanol + 1 ml developer) and purified water were tested to read 0 ppb on a calibrated fluorometer.

Sample extraction: About 50 g ground sample with 5 g NaCl was placed in a blender jar and blended with 100 ml methanol: water at high speed for 1 minute. The extract was poured into clean vessel through fluted filter paper.

Dilution and filtration: Ten ml. of filtered extract was diluted with 40 ml of purified water and passed through glass microfibre filter into glass syringe barrel.

Affinity chromatography: Two ml of filtered diluted extract was passed through afaltest affinity column at a rate of 1-2 drops/ seconds, until air came out of the column. Passed 5 ml of purified water through the column and repeated until air came out of the column. The column was then eluted by passing 1 ml of HPLC grade methanol and collected in a glass cuvette.

Added 1 ml of aflatest developer to eluate in the cuvette, mixed well and placed in the calibrated fluorometer and read the aflatoxin concentration after 60 seconds.

3.5. Growth and feed performance in *P.monodon* postlarvae fed different doses of AFB_1

Three sets of experiments were conducted in different stages of postlarvae.

3.5.A. A feeding trial of 30 days duration was conducted to study the effects of aflatoxin B_1 on *P.monodon* postlarvae to study weight gain, survival and histological changes. The doses of aflatoxin selected were 0 (control), 20 ppb, 50 ppb, 2500 ppb and 5000 ppb (Table 3.3). A shrimp feed containing crude protein 41% was formulated to make the experimental diets. 100 g of feed was prepared for the control and each of the treatments. Dry powdered feed ingredients were weighed and mixed manually to achieve uniform mixing. After steaming, vitamins, minerals, oil and toxin dissolved in alcohol were added and mixed thoroughly. The dough after moistening was pelleted through a 1 mm die into separate trays. The pellets were dried in oven at 60°C and the dried pellets were stored in airtight bottles.

Table 3.2. Ingredient composition of feed

Ingredients	Weight g
Fish meal	18
Shrimp meal	18
Clam meal	12
Soyabean meal	18
Wheat flour	18
Vitamins	2
Minerals	3
Oil[§]	5
Lecithin	2
Gelatin	4
Total	100

Vitamin premix (mg or IU /kg in diet) :
Vit.A - 6000 I.U.; Cholecalciferol - 1500I.U.; Tocopherol acetate- 150 I.U.; Menadione- 20 mg.; Ascorbic acid- 200 mg.;Thiamine hydrochloride - 60 mg.; Riboflavin – 40 mg.; Calcium pantothenate- 60 mg.; Pyridoxine hydrochloride – 40 mg.; Nicotinic acid – 200 mg.; D-Biotin – 1 mg.; Choline chloride – 500 mg.; Inositol- 250 mg.

Mineral premix (g/Kg in diet) :
$CaHPO_4.2H_2O$-8g;Mg $SO_4.7H_2O$- 5g; KH_2PO_4- 4g; Na_2 $H_2PO_4.2H_2O$-2 g; $MnSO_4.H_2O$- 0.6g; $FeSO_4$ –0.6g; $ZnSO_4.7H_2O$-0.6 g; $CO(NO_3)_3$ - 0.1 g; $CuSO_4.5H_2O$-0.1g

Oil[§] : A combination of 1:1 cod liver oil and vegetable oil

3.5.1. Experiment protocol

Penaeus monodon postlarvae of size 0.06 ± 0.013 g were obtained from a hatchery at Malipuram, Ernakulam. The postlarvae were slowly acclimatized to laboratory conditions of 20 ppt salinity, 26-± 2°C temperature and dissolved oxygen 5 ± 1 ml/l and fed the control diet. They were sorted into groups of 12 numbers so that triplicates were available for

each of the three treatments and the control. Shrimps were stocked in 40 l plastic tubs containing 25 l seawater. The plastic tubs were arranged vertically on racks and each tub was provided with aeration . 50% water was exchanged daily morning, while complete water change was done every third day.

Experimental diets were fed at the rate of 15% of the initial body weight of the postlarvae for the first 15 days and 12 % for the next 15 days. Daily feed ration was divided into 3 doses and fed at 0900 hours (30%), 1400 hours (20%) and 1800 hours (50%). Tubs were cleaned before each feeding. Every fortnight, the animals were weighed. Mortality and feeding behaviour was recorded regularly. After 30 days the experiment was terminated and the animals in each tub weighed. Based on the results obtained, the AFB_1 doses for the subsequent experiments were selected.

Table 3.3. Dosages of AFB_1 in feed for the first experiment on *P. monodon* postlarvae

Doses of AFB_1	Working Solution	AFB_1 in feed Per 100 g.
20 ppb=20 µg/kg	0.01 ml	2 µg
50 ppb=50µg/kg	0.025 ml	5µg
2500 ppb=2.5 mg/kg	1.25 ml	250µg
5000 ppb=5 mg/kg	2.5 ml	500µg

3.5.B. For the second experiment, the size of postlarvae was 0.056 ± 0.001 g and the trial was conducted for 45 days to study the effects of aflatoxin B_1 on weight gain, specific growth rate, apparent food conversion and survival rate. The doses of AFB_1 selected were 50 ppb, 250 ppb, 500 ppb, 750 ppb, 1000 ppb and 2000 ppb (Table 3.4). Experimental diets were fed at the rate of 15% of their initial body weight for the first 15 days, 12 % for the next 15 days and 10% for the last 15 days.

Table 3.4. AFB_1 dosages in feed in the second experiment on *P. monodon* postlarvae

Doses of AFB_1	Working solution (ml)	AFB_1 in feed per 100 g
50 ppb	0.025	5µg
250 ppb	0.125	25 µg
500 ppb	0.25	50µg
750 ppb	0.375	75µg
1000 ppb	0.5	100 µg
2000 ppb	1	200 µg

3.5.C. For the third experiment on growth and feed performance *Penaeus monodon* postlarvae of size 0.11 ± 0.02 g were obtained from Cochin Aqua hatchery at Malipuram, Ernakulam. The postlarvae were acclimatized to laboratory conditions of salinity 20±1 ppt, temperature 26 ± 2°C and dissolved oxygen 5 ± 1 ml/l for 10 days and fed the control diet. They were sorted into groups of 15 numbers so that triplicates were available for each of the three treatments and the control (Plate 1a). Postlarvae were stocked at 15 nos. in 50 l plastic tubs containing 40 l seawater. The plastic tubs were arranged vertically on racks and each tub was provided with aeration. Water exchange was at 50% daily while complete water change was done every third day. Doses selected for the study were 0, 50 ppb, 500ppb, and 2000 ppb (Table 3.5). Three diets with the selected doses of AFB_1 and one control (diet without toxin) were formulated and 100 g. of feed was prepared for each of the treatments and the control for 45 days experiment. The feed composition is presented in Table 3.2.

Experimental diets were fed at the rate of 15% of the initial body weight for the first 15 days, 12 % for the next 15 days and 10% for the last 15 days. Daily feed ration was divided into 3 doses at 0900 hours (30%),

1400 hours (30%) and 1800 (40%) hours daily. From day 5 to day 40, faecal matter and left over feed were collected from each tub every morning with the help of a plastic tubing, rinsed with distilled water and dried at 70 °C for 12 h and pooled for analysis later. Every fortnight, the shrimps were weighed and the feeding rate was adjusted. Mortality and feeding behaviour were recorded regularly. After 45 days the experiment was terminated and the shrimps in each tub were weighed and their proximate composition determined.

Table 3.5. Dosages of AFB_1 in feed for study on growth responses in postlarvae

Doses of AFB_1	Working Solution	AFB_1 in feed Per 100 g.
50 ppb= 50µg/kg	0.025 ml	5µg
500 ppb=0.5mg/kg	0.25 ml	50µg
2000 ppb= 2mg/kg	1 ml	200µg

3.5.2. Data collection

Each of the tubs was provided with a clipbord indicating the dose of aflatoxin and other details of the experiment for record maintenance. The tubs were monitored daily for any unusual behaviour and feeding activity. The data collected from each tub were feed given and consumption; mortality and water parameters like salinity, temperature and dissolved oxygen. The performance parameters considered were weight gain, apparent feed conversion ratio, specific growth rate, percentage survival, protein efficiency ratio, net protein retention, apparent feed digestibility and protein digestibility. At the end of the experiment, three postlarvae were fixed with Davidson's fixative and the cephalothorax was observed for histological changes.

3.5.3. Formulae used

1. **Weight gain %** = $\frac{\text{Final wt} - \text{Initial wt.}}{\text{Initial wt}}$

2. **Specific growth rate** = $\frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{\text{No. of days of experiment}} \times 100$

3. **Apparent feed conversion ratio** = $\frac{\text{Feed intake, g dry weight}}{\text{Weight gain, g wet weight}}$

4. **Feed digestibility** = $\frac{\text{Amount of feed consumed} - \text{Amount of faecal matter}}{\text{Amount of feed consumed}}$

5. **Protein digestibility** = $\frac{\text{Protein given in feed} - \text{Protein in faecal matter}}{\text{Protein given in feed}}$

6. **Protein efficiency ratio** = $\frac{\text{Shrimp wet weight gain g}}{\text{Protein intake}}$

7. **Net protein retention** = $\frac{\text{Final body protein} - \text{initial body protein}}{\text{Protein intake}}$

8. **Survival** = $\frac{\text{Final no. of shrimps}}{\text{Initial no. of shrimps}} \times 100$

3.6. Effect of dietary AFB_1 with sub-lethal levels of copper/cadmium in water on *P. monodon* postlarvae

The combined effect of heavy metal like copper and cadmium and aflatoxin was studied in *P. monodon* postlarvae

3.6.1. Determination of 96 hour LC_{50} of copper (Cu) and cadmium (Cd) in *P. monodon*

Copper test solutions were prepared by dissolving 5g of copper sulphate in 20 ml of distilled water to prepare 1000 mg/l copper stock solution and then diluted with seawater of 20 ppt salinity to make working solutions containing 0, 2, 4, 6, and 8 mg/l for determination of 96 hour LC_{50} of copper in *P.monodon*

From previous reports, the LC_{50} value of cadmium for PL25 of *P. monodon* was found to be 1.78 mg/l (Munshi *et al.*, 1997). Hence the test concentrations of cadmium chloride were prepared by diluting 2g of

cadmium chloride in 10 ml of distilled water and diluted with seawater of 20 ppt salinity to make 0, 0.75, 1.5, 2.25 and 3.0 mg/l cadmium.

Lethal effect of copper and cadmium

LC_{50} toxicity was determined according to the method described by APHA (1985). Static bioassay experiments were conducted in 20 l polythene tubs containing 10 l of test solution. Each tub contained 10 shrimp. There were triplicates for each test solution with a total number of 30 postlarvae. Each test solution was renewed daily morning at 0900 hrs. During the experiments the shrimps were fed a control diet as in other experiments twice a day (0900 hours and 2000 hours) at 15 % of body weight. Observations were made at 24 hours interval upto 96 hours. Death was assumed when shrimps were immobile and showed no response when touched with a glass rod. The response curve was computed using Microsoft Excel software to determine 96 hour LC_{50} of Cu and Cd in *P.monodon.* The 'best fits' were drawn based on the regression equation ($y = a + bx$) between Cu and Cd concentration (as X) and percentage mortality of shrimps (as Y) after 96 hours (Mohapatra and Noble, 1991)

3.6.2. Experimental Design

Postlarvae of size 0.55 ± 0.04 g were obtained from a hatchery at Malipuram, Ernakulam. Prior to initiation of the experiment, the postlarvae were slowly acclimatized to laboratory conditions for 10 days and fed the control diet. The PL were stocked at the rate of 10 numbers in 45 l plastic tubs containing 30 l of seawater. There were triplicates for each test solution with a total number of 30 PL for each group (Plate 1b). Three treatments and one control were taken for the study (Table 3.6). The control group was given normal feed. The second group was given 500 ppb AFB_1 diet. The third and fourth group were exposed to sub-lethal levels of copper/ cadmium

Plate 1

Plate 1a. Set up for the experiment on growth performance in *P. monodon* postlarvae

Plate 1b. Set up for the experiment on interactive studies in *P. monodon* postlarvae

in the rearing water and fed 500 ppb aflatoxin B_1 diet. Stock solutions of copper sulphate and cadmium chloride were prepared and the exact amount of test solution was added to 30 l of plastic tubs. The plastic tubs were arranged vertically on racks and each tub was provided with aeration. The parameters maintained were 20 ppt salinity, 26 ± 2°C temperature and dissolved oxygen 5 ± 2 ml/l for the entire experimental duration. Water exchange was 50% daily, while complete water change was done every third day. The appropriate levels of copper/cadmium were maintained accordingly.

Experimental shrimps were fed at the rate of 15% for the first 15 days, 12 % for the next 15 days and 10% for the last 10 days. Daily feed ration was divided into 3 doses and fed at 0900, 1400 and 1800 hours. From day 5 to day 40, faecal matter and left over feed were collected from each tub every morning with the help of a plastic tubing, rinsed with distilled water and dried at 70°C for 12 h and pooled for analysis later. Every fortnight, the shrimps were weighed and the feeding rate was adjusted accordingly. Mortality and feeding behaviour was recorded regularly. After 40 days the experiment was terminated and the shrimps in each tub weighed.

3.6.3. Data collection

The growth parameters observed were increase in weight, percentage weight gain, specific growth rate, apparent feed conversion ratio, survival, protein efficiency ratio and net protein utilization.

Table 3.6. Dosage of AFB_1 and Cu /Cd selected for interaction study in postlarvae

Groups	Doses of AFB_1	Sub-lethal levels
Control	-	-
AFB_1	500 ppb	-
Cu+ AFB_1	500 ppb	Copper (0.526 ppm)
Cd+AFB_1	500 ppb	Cadmium (0.192 ppm)

3.7. Pathological and immunological changes in *P. monodon* fed different doses of AFB_1 in diets.

3.7.1. Experiment protocol

Penaeus monodon sub-adults of size 7.5 ± 0.72 g brought from a farm at Narakkal, Ernakulam district, Kerala were acclimatized to 20 ppt salinity for one-week in the holding tanks of 2-ton capacity. One control and six treatment groups were selected for the experiment of 60 days duration. The doses of aflatoxin selected were 0, 50 ppb, 100 ppb, 150 ppb, 500 ppb, 1000 ppb and 2000 ppb (Table 3.7). Shrimps were weighed and about 26 nos. segregated into separate 1-ton FRP tanks of 2 m length, 1m width and 0.5 m depth (Plate 2a). Feeding rate was 4.2% for the first 30 days and 3.5% for next 30 days. Shrimp feed formulation containing 38% crude protein was used to make experimental diets. Small amounts of feed were added at a time to ensure complete feeding to avoid feed wastage and contamination. Water exchange was carried out daily at 50% level. Water quality, temperature, dissolved oxygen and salinity were monitored daily. The shrimps were observed for any unusual behaviour and morphological changes. About 15 nos. of shrimps were sacrificed for determining normal haematological parameters at the beginning of the experiment. Three shrimps from each group were taken for histological and ultrastructural study. At the end of the experimental duration, the shrimps from each group were processed for aflatoxin residue analysis. After 30 days, 13 shrimps

were harvested from each treatment group and at the end of 60 days the remaining 13 shrimps from each treatment group were taken for analysis.

Table 3.7.Dosage of AFB_1 in shrimp feeds for study on pathological and immunological changes in *P. monodon*

Doses of AFB_1	Working Solution of AFB_1	AFB_1 in feedper 500g.
50 ppb = 50µg/kg	0.125 ml	25µg
100 ppb=100µg/kg	0.25 ml	50µg
150 ppb =150µg/kg	0.375 ml	75µg
500 ppb = 0.5µg/kg	1.25 ml	250µg
1000 ppb= 1mg/kg	2.5 ml	500µg
2000 ppb= 2mg/kg	5 ml	1000µg

Table 3.8. Feed formulations for sub-adults of *P. monodon*

Ingredients	Weight g
Fish meal	14
Shrimp meal	14
Clam meal	12
Soyabean meal	20
Wheat flour	24
Vitamins	2
Minerals	3
Oil[§]	5
Lecithin	2
Gelatin	4
Total	100

Vitamin premix (mg or IU /kg in diet) :

Vit.A - 6000 I.U.; Cholecalciferol - 1500I.U.; Tocopherol acetate- 150 I.U.; Menadione- 20 mg.; Ascorbic acid- 200 mg.;Thiamine hydrochloride - 60 mg.; Riboflavin – 40 mg.; Calcium pantothenate- 60 mg.; Pyridoxine hydrochloride – 40 mg.; Nicotinic acid – 200 mg.; D-Biotin – 1 mg.; Choline chloride – 500 mg.; Inositol- 250 mg.

Mineral premix (g/Kg in diet)

$CaHPO_4.2H_2O$-8g; $Mg\ SO_4.7H_2O$- 5g; KH_2PO_4- 4g; $Na_2\ H_2PO_4.2H_2O$- 2 g; $MnSO_4.H_2O$- 0.6g; $FeSO_4$ –0.6g; $ZnSO_4.7H_2O$-0.6 g; $CO(NO_3)_3$ - 0.1 g; $CuSO_4.5H_2O$-0.1g

Oil[§] : A combination of 1:1 cod liver oil and vegetable oil

3.7.2. Feed preparation

All the feed ingredients (Table 3.8) were taken to prepare 7 types of AFB_1 diets (Table 3.7).

3.7.3. Data collection

The tanks were labeled with clipboard indicating details of experiment such as dose of aflatoxin and day of experiment for record maintenance. The tanks were monitored daily for any unusual behaviour and feeding activity. The data collected from each group and replicate tanks were feed given and consumption, mortality, and salinity, temperature and dissolved oxygen in the water. The haemolymph collected from shrimps under different treatment and control were observed for cellular factors like total haemocyte count, differential haemocyte count and phagocytosis, humoral factors like phenoloxidase, acid phosphatase, alkaline phosphatase, total serum protein, glucose, and cholesterol. The cephalothorax was taken for histological study. The hepatopancreas from all the treatment groups were observed for ultrastructural changes. Shrimp samples from each group were analysed for toxin residues.

Haemolymph collections were made from the pericardial cavity of each shrimp using 1cc/2cc disposable syringe fitted with 22g, 3.8 cm needles. The needle was inserted through the intersegmental membrane between the cephalothorax and the abdominal segment of the shrimps to reach the pericardial region and haemolymph was withdrawn. Care was taken not to extract tissue particles with the haemolymph. The haemolymph samples from each treatment group were pooled and taken in three separate lots of eppendorf tubes of 0.5 ml capacity for haemocyte count, serum extraction and lysate preparation.

3.7.3.1. Haemolymph for haemocyte count and phagocytosis

The first lot of haemolymph collected in a syringe was added directly into eppendorf tubes containing 10% cold seawater formalin for estimating total haemocyte count and differential haemocyte count. For phagocytosis, haemolymph taken in syringe was added directly into eppendorf tubes containing M199 culture media and buffer.

3.7.3.2. Serum preparation

The second lot of pooled haemolymph samples taken in 0.5ml eppendorf tubes was allowed to clot at ambient temperature (27°C) for 4 hours after extraction. Serum was obtained by centrifuging at 12500 g for 10 minutes and then at 20000 g for 5 minutes. After centrifuging, the clear, bluish supernatant was pipetted into individual vials and stored at –18 °C until further analysis.

3.7.3.3. Lysate preparation

The third lot of pooled haemolymph samples taken in 0.5 ml eppendorf tubes was refrigerated for 3 hours. The clotted haemolymph samples were macerated in a tissue homogeniser placed in a beaker of ice to break the clot and centrifuged at 10,000 rpm for 5 minutes. The supernatant formed the lysate for further study.

Response parameters

1. Total haemocyte count (THC)

THC was determined following the method of Perazzolo and Barracco, (1997). Cold seawater formalin (10%) (Mix and Sparks, 1980) was used as fixative to draw haemolymph. Seawater of 35‰ salinity was filtered and autoclaved at 15 lb pressure for 15 minutes. One ml of formalin (34%) was added to 9 ml of filtered seawater to form 10% seawater

formalin and cooled in refrigerator before haemolymph extraction. Two ml. of the fixative was taken in 0.5 ml of eppendorf tube and 0.1 ml of haemolymph sample added to eppendorf tubes.

The haemolymph samples from each treatment group were taken in triplicate. The tubes were shaken for 2 minutes in a vortex shaker for uniform blending of haemocytes and the fixative. After mixing, 0.1 ml of the haemolymph samples from each eppendorf tube were loaded in a newbauer haemocytometer and viewed under phase contrast microscope (Ceti Model). The total numbers of haemocytes in the four large corners of the haemocytometer were counted (1group square =16 small squares). The total haemocyte count were obtained by multiplying the total number of cells with dilution factor and divided by volume of the counting chamber. The dilution factor obtained from the amount of diluent (fixative) and haemolymph added. The volume was obtained from area x height of the counting chamber. THC expressed as count x 10^4 cells/ml.

$$\text{Calculations} = \frac{\text{Total no. of cells (N) x Dilution. Factor(df)}}{\text{Volume of the counting chamber}}$$

$$\text{Dilution factor (df)} = \frac{\text{diluent(ml) + haemolymph (ml)}}{\text{haemolymph (ml)}}$$

$$\frac{N \times df}{\text{Area} \times \text{depth}} = \frac{N \times df}{0.4} \times 10^3/\text{ml} = N \times 10^4 \text{ cells/ml}$$

2. Differential haemocyte count (DHC)

The method of Mix and Sparks (1980) was followed for determining DHC. Cold seawater formalin (10%) was used as fixative in a 2 ml syringe with 26-gauge needle to draw haemolymph. Haemolymph (0.1 ml) and seawater formalin (0.2 ml) were slighty mixed in a glass vial and refrigerated for 1-3 hours. After fixation, the haemocytes were concentrated by centrifugation at 8000 rpm for 4 min at 4°C. Smears were made on

alcohol clean slides from the concentrate and allowed to dry. After drying, slides were fixed with methanol for 2-3 minutes. Wright's stain diluted with phosphate buffer (pH 7.2) in 1:1 ratio and filtered through Whatman No.1 filter paper was used for staining the slides for 5 minutes. The scum formed on the slides was washed with water, dried and examined under oil-immersion objective of the microscope. About 100 numbers of haemocytes were counted from each slide and classified into three types.

A. Hyalinocyte -cell with blue nucleus and almost visible clear cytoplasm.

B. Semigranulocyte-cell with comparatively larger blue nucleus and cytoplasm containing granules. The granules range from blue to dark blue and acidophilic red.

C. Granulocyte- cell with blue nucleus and large number of acidophilic granules that make the cytoplasm seem very red and more conspicuous.

The means from each treatment group were averaged from three glass slides.

3. Phagocytosis

The method of Itami *et al.* (1994) was modified for determination of percentage phagocytosis. M-199 Culture media was prepared to estimate the phagocytosis of hemocytes and Citrate/EDTA buffer was used as anticoagulant. Both the buffer and the media were chilled before use (0.5 ml+0.5 ml). About 0.1 ml of haemolymph was transferred to 1 ml chilled media in an eppendorf tube, and centrifuged at 8500-9000 rpm for 10 min at -15°C. The supernatant was decanted and the pellet agitated. Of this about 10-50 µl was spread on clean dry coverslip and incubated at 25°C for 30 minutes. Formalin killed yeast cells given final wash with medium were layered over the coverslip and incubated for 1 hour at 25°C in a moist CO_2 chamber. Microslides were washed and then fixed with methanol for 5 minutes and then stained with Wright-Giemsa stain. After mounting in

DPX, the micro slides were observed under the oil immersion microscope. The total number of phagocytic cells with engulfed yeast cell was counted and the phagocyte ratio calculated. The means for each treatment group were calculated from three microslides.

$$\text{Phagocytosis} = \frac{\text{No. of phagocyte cells with engulfed yeast cells}}{\text{No.of phagocytes}} \times 100$$

4. Phenoloxidase activity was determined following a modified method of Preston and Taylor (1980). The haemolymph lysate was used as sample and 0.01M Dopa (Diphenyl hydroxyphenylalanine) in 0.05 M Tris HCl buffer at pH 7.5 formed the substrate .To activate the enzyme, few crystals of sodium dodecyl sulphate (SDS) were added to the substrate. To two ml of the substrate, 0.2 ml serum was added and the optical density was noted immediately at 490 nm in a spectrophotometer up to 3 minutes at 30 sec interval. Prepared the control with 0.2 ml of distilled water in 2 ml of substrate. The protein content of the sample was determined by the method of Lowry *et al.* (1951) and the results expressed as OD/mg protein/min.

5. Alkaline phosphatase (ALP). Kind and King (1954) method was used for estimating ALP. Two ml of buffered substrate (pH 10) was pippeted into 2 tubes marked as test and control and incubated for a few minutes at 37°C. Added 0.1 ml serum to test. Again incubated at 37°C for 15 min. after which the tubes were removed from the waterbath. Added 0.8 ml of NaOH and 1.2 ml of $NaHCO_3$ to both test tubes. Then added 0.1 ml of serum to control, followed by addition of 1 ml of 4-aminoantipyrine and 1 ml of potassium ferricyanide to both tubes and read the absorbance at 520 nm. For Standard, 1 ml of working standard and 1.1 ml of buffer were added, followed by the other reagents as in test. For the blank, 1 ml of distilled water and 1.1 ml

buffer and other reagents as in test were added and the values expressed as K.A.units (mg phenol liberated / 100 ml serum/hr).

$$\text{Alkaline phosphatase in K.A units} = \frac{\text{OD of test} - \text{OD of control}}{\text{OD of standard} - \text{OD of blank}}$$

6. Acid phosphatase (ACP) was determined following the method of Kind and King (1954). The method for determination was similar to that of alkaline phosphatase except for the buffered substrate being at pH 4.9 and incubated for 1 hour at 37 °C. Values were expressed as K.A.units (mg phenol liberated / 100ml serum /hr).

$$\text{Acid phosphatase in K.A units} = \frac{\text{OD of test} - \text{OD of control}}{\text{OD of standard} - \text{OD of blank}}$$

7. Total serum protein was determined following Lowry *et al.* (1951). To 1 ml of deproteinizing agent (80% ethanol) 0.05 ml serum was added, centrifuged at 3000 rpm for 5 minutes, and decanted the supernatant. Dissolved the precipitate in 1 ml of NaOH and 5 ml of alkaline copper reagent and mixed well. After 10 minutes added 0.5 ml folin phenol regent and mixed well. The blank was set up without the sample. After 30 minutes measured the optical density of the blue colour developed by the sample at 500 nm. Calculated the protein concentration by referring the OD obtained for sample using standard graph. Total serum protein was expressed asg/100ml.

8. Serum Glucose was anlaysed as per the modified method of Hugget and Nixon (1957). To 0.1 ml of serum, 0.4 ml of 5% $ZnSO_4.7H_2O$, 0.4 ml of 0.3 N NaOH and 1.1 ml of 0. 9% NaCl were added; mixed well, centrifuged, and the supernatant separated as soon as possible. Transferred 1 ml of the

supernatant to test tube marked as test. One ml of water taken as blank and 1 ml of standard glucose solution was taken as standard. Added 3 ml of glucose oxidase reagent to all the test tubes at half-minute interval, mixed gently for not more than 10 seconds, and read the absorbance in a spectrophotometer at 625 nm. The values were expressed as mg glucose /100 ml.

$$\text{Glucose} = \frac{\text{OD of sample x conc. of standard x dilution factor x 100}}{\text{OD of standard}}$$

9. Serum cholesterol

Cholesterol was estimated according to the method of Varley (1975). About 0.5 ml to 2. 5 ml of working cholesterol solution was pippeted out into clean test tubes. The total volume of each tube was made upto 5 ml with ferric chloride diluting reagent. To 0.1 ml of serum, 4.9 ml of ferric chloride precipitating reagent was added and mixed well. Allowed to stand for 5 minutes and centrifuged. Transferred 2.5 ml of clear supernatant into dry test tubes, added 2.5 ml of ferric chloride diluting reagent, and mixed well. Tubes were kept in cold water and to each tube 4 ml of Conc.H_2SO_4 was added, mixed well and brought to room temperature. A blank was also simultaneously prepared by taking 5 ml of diluting reagent and 4 ml of Conc. H_2SO_4. After 30 min, the intensity of colour developed was read at 540 nm against blank. The values were expressed as mg/100 ml.

$$\text{Cholesterol mg /100 ml} = \frac{\text{OD of sample x Conc. of standard}}{\text{OD of standard}}$$

10. Histology

The cephalothorax region and hepatopancreas of *P.monodon* were taken for study.

Fixation: The tissues were fixed in Davidson's fixative overnight. The tissues were scored with a sharp blade for the easy penetration of the fixative.

Tissue processing and microtomy: Tissues were processed for paraffin embedding in an automatic tissue processor (Leica, Germany) and sections of 5-6µm thickness were cut in a semi-automatic rotary microtome.

Staining: The paraffin sections taken on glass slides were cleared in xylene, hydrated with descending grades of alcohol, stained in haematoxylin; passed through acid alcohol, Scott's top water and then stained by eosin. The stained sections were dehydrated in ascending grades of alcohol, cleared in xylene before mounting with DPX and observed under light microscope (Leica DMLS).

11. Electron microscope

The ultrastructural studies were carried out in Hitachi - H-600 Transmission Electron microscope (Hitachi Ltd.,Tokyo), The tissue preparation and processing were done as described by Dawes (1988).

Primary fixation: One mm-sized pieces of hepatopancreas were collected and immediately transferred to chilled 3% glutaraldehyde (modified sucrose buffer pH 7.4). The tissues were kept under refrigeration until trimmed. Each piece of tissue was kept on a wooden plank with a drop of 3% glutaraldehyde and cut into pinhead sizes with a sharp surgical blade and again transferred to chilled fixative, and stored at 4 ° C for 24 hours.

Washing and Post-fixation: The tissues were washed in 0.1% cacodylate buffer thrice (each 15 min) and post-fixed in 0.1% osmium tetroxide for 1 hour until it became black/brown colour. Again washed three times with 0.1% cacodylate buffer (25 min each).

Dehydration: Carried out in ascending grades of acetone (Analar) at 4°C and embedded in Spurr's resin as per the method described by Spurr (1969).

After infiltration and embedding, the samples were taken in a bean capsule filled with spur and oriented to bottom avoiding air bubbles and kept in BOD incubator at 70°C for 12-14 hrs.

Sectioning: Ultra-thin sections were taken in LKB Nova Ultra microtome (CLKB – Producter AB, Sweden). These sections were lifted on the matted surface of copper grid having 100-300 mesh sizes. These grids with the sections were stained with uranyl acetate and lead nitrate stains (Spurr,1969). Further the grids were dried and observed under transmission electron microscope.

12. Analysis of AFB_1 residue in muscle (AOAC, 1990)

At the end of the experiment, shrimps from each group were oven-dried at 70°C for 2 days and powdered finely for determination of AFB_1 residue in the body of shrimps.

Processing: The powdered samples were weighed and mixed homogenously and taken into 500 ml conical flasks with 25 ml water and 250ml chloroform ($CHCl_3$). The flasks were shaken for 30 minutes to extract the toxin and filtered through whatman No.41 filter paper.

Removal of interfering substances by column chromatography

The chromatographic column consisted of a glass tube 22 x 300 mm with a glass stopper at the bottom. The column was filled with anhydrous Na_2SO_4, silica gel, and chloroform, and 50 ml filtered extract was added to the column. After draining the $CHCl_3$, the column was washed with hexane and diethyl ether. Finally, the aflatoxins were eluted from the column with methanol–chloroform mixture. The elute was evaporated in a vacuum flash evaporator and the residue was dissolved in a known volume of $CHCl_3$.

Separation of AFB_1 by TLC

The standard solution containing known quantities of AFB_1 were spotted along with sample extracts in a thin layer chromatography plate. Plates were developed ascendingly by dipping in a chromatographic tank containing chloroform-acetone. After development, plates were dried and viewed in UV cabinet. Blue fluorescence colour indicated the presence of AFB_1. Rf values of 0.5 to 0.55 corresponded to AFB_1.

Quantitative analysis of AFB_1

The area covering the spots were marked by a sharp needle and the silica gel covering each spot was scrapped with a blade and collected in a clean dry test tube. The toxins were extracted with 2ml methanol for 3 min, washed thrice with methanol and the combined filtrate made up to 3 ml. Aflatoxin content was determined by measuring the absorbance at 360 nm. The amount of aflatoxin present in the unknown was computed from the formula.

$$AFB_1 \text{ content (ppb)} = \frac{S \times V \times 5}{A \times W}$$

Where W= weight of original sample, V = µl of sample extract prepared for TLC, A= µl of sample extract spotted, S = µl of AF calculated from standard curve.

3.8. Amelioration of AFB_1 toxicity

3.8.1. Experimental design

The efficacy of a spicy herbal powder, Amrita Bindu, (AB), Vitamin E (a- tocopherol acetate) and Vitamin K (Menadione sulphate) in ameliorating the toxicity of AFB_1 was studied in *P. monodon* sub-adults. Amrita Bindu, a herbal medicine successfully used in detoxification of AFB_1 in rats and fishes was used for amelioration in the present study (AB

was gift from Prof.E.R.B. Shanmugasundaram Herbal Research foundation, Chennai formulated by Shanmugasundaram *et al.*, 1994).

3.8.2. Experimental animals

About 120 numbers of *P.monodon* sub-adults of size 11.3 ± 1.24 g were obtained from Matsyafed shrimp farm, Vypeen, Ernakulam District, Kerala. The shrimps were transferred by hand-nets to holding tanks and slowly acclimatized to 20 ppt salinity, 26 ±2 °C temperature and 5 ±1 mg/l dissolved oxygen for 10 days prior to the experiment. Twenty sub-adult shrimps were stocked in 1-ton FRP tanks for each group (Plate 2b).

3.8.3. Feed preparation

Dry feed ingredients, fish meal, shrimp meal, soyabean meal, clam meal and wheat flour, were used for preparing 500 g feed for each of the eight treatment groups for 40 days duration. Diet 1 prepared was normal shrimp feed of 38% crude protein. Diet 2 was prepared by adding 500 ppb of AFB_1 to the oil portion of the diet 1. Diet 3 was prepared by adding 4 % (4g/100g)of the spicy herbal powder along with 500 ppb AFB_1. Since this was first attempt in shrimps, the powder was fed in lower doses to monitor their acceptance for a week prior to the experiment. Diet 4was prepared by adding 4 g% Amrita Bindu to the diet 1. The Vitamin E requirement by shrimps being 200 IU /Kg (Paul raj, 1993), the diet 5 was prepared by adding about five times the normal requirement of Vitamin E (1000 IU/Kg) along with 500 ppb AFB_1. Diet 6 was prepared by adding 1000 IU/kg Vitamin E to the diet 1. The Vitamin K requirement of shrimps in feed being 20 mg/kg (Paul raj, 1993), diet 7 was prepared by mixing 100 mg/Kg Vitamin K along with 500 ppb toxin in the diet 1. Diet 8 was prepared by adding 100 mg/Kg Vitamin K. Eight different test diets were prepared as pellets and stored in covered airtight bottles. The proximate composition of

Plate 2a . Set up for the experiment on pathological and immunological changes in *P. monodon* juveniles fed aflatoxin B_1 incorporated diets

Plate 2b. Set up for the experiment on amelioration of aflatoxin B_1 toxicity in *P. monodon* sub-adults

feed ingredients and feed formulation were same as in experiment 3. Shrimps were fed the experimental diets at 2-4% level. The daily feed ration was split into three and fed at 0900 hours (3%), 1400 hours (2%) and 1800 hours (5%).

The tanks were checked daily for feeding activity, morphological changes, mortality, salinity, temperature, and alternate days for dissolved oxygen and ammonia. Shrimps were sampled at the end of 4 weeks for analysis. Haemolymph samples from each treatment group were pooled for total haemocyte count and serum extraction. The cephalothorax region of shrimps from each group was fixed in Davidson's fixative for histological studies. The shrimps were dried and powdered for detection of toxic residues by TLC method.

Table 3.9. Composition of the eight diets used for AFB_1 amelioration study in *P. monodon*

Groups	Diets	AFB_1 levels	Additives used for Amelioration
Group 1	Diet 1	-	-
Group 2	Diet 2	500 ppb AFB_1	-
Group 3	Diet 3	500 ppb AFB_1	4 % AB
Group 4	Diet 4	-	4 % AB
Group 5	Diet 5	500 ppb AFB_1	1000 IU/kg Vit. E
Group 6	Diet 6	-	1000 IU/kg Vit. E
Group 7	Diet 7	500 ppb AFB_1	100 mg/kg Vit.K
Group 8	Diet 8	-	100 mg/kg Vit.K

3.8.4. Analysis of haemolymph

The tanks were labeled with clipboard indicating dose of aflatoxin and day of experiment for record maintenance. The tanks were monitored daily for any unusual behaviour and feeding activity. The data collected from the experiment were feed given and consumption, mortality and water parameters like salinity, temperature and dissolved oxygen.

Haemolymph collections were made from the pericardial cavity of each shrimp using 1cc/2cc disposable syringe fitted with 22g, 3.8 cm needles. External water present on the shrimps was removed prior to haemolymph collection. The needle was inserted through the intersegmental membrane between the cephalothorax and the abdominal segment to reach the pericardial region and as much haemolymph was withdrawn from each shrimp. Care was taken not to extract tissue particles with the haemolymph .The haemolymph collected from shrimps were observed for cellular factor like total haemocyte count, and the serum was used for determination of parameters like acid phosphatase activity, alkaline phosphatase activity, total protein, albumin and globulin levels, glucose levels, cholesterol, triglycerides levels, aspartate transaminase, alanine transaminase, and lactate dehydrogenase activity. The cephalothorax of shrimps from each group was studied for histological changes. The shrimp carcass was analysed for retention of AFB_1 residues.

Haemolymph samples were allowed to clot at ambient temperature (27°C) for 4 hours after extraction. Serum was obtained by centrifuging haemolymph samples at 12500 x g for 10 minutes and then at 20000 x g for 5 minutes. After centrifuging, the clear, bluish supernatant was pipetted into individual vials and stored at –18 °C until analysis.

Total Protein and Albumin were determined by the **(Biuret method)** of Doumass *et al.* (1971)

Total protein

1.9 ml of 0.9% saline was added to a test tube containing serum (0.1 ml) and mixed by inversion. To the solution added 5 ml of biuret reagent and all the tubes were shaken well and placed in a water bath at 37°C for 10 min, allowed to cool to room temperature for 5 min, and then read the absorbance at 555 nm.

Total Albumin

Pippeted 5.7 ml of 28% Na_2SO_4 solution into a centrifuge tube and added 0.3 ml of serum . Rotated the tubes gently between the palms and 3 ml of ether was added and the tube was gently shaken upside down for about 20 times. Waited for 10 minutes till a globulin button formed at the interphase of both saline. Centrifuged for 10 minutes to complete the process of globulin button formation and hardening. After centrifuging, tilted the tube and inserted a pippete into clear solution below the globulin layer, without disturbing the precipitate. About 2 ml of this solution was added to 5 ml of biuret reagent. Different concentrations of the standard ranging from 0.2 ml to 1.4 ml was pippeted into clean dry test tubes and made upto 2 ml with 0.9% saline. Added 5 ml of biuret reagent and all the tubes were shaken well and placed in a water bath at 37° C for 10 min, allowed to cool to room temperature for 5 min, and read the absorbance at 555 nm. The difference between total protein and albumin gives globulin values. The values were expressed as g/100ml.

Globulins in gm% =Total proteins in g% - albumin in g %

Albumin/ Globulin ratio = $\frac{\text{Albumin in g\%}}{\text{Globulins in g\%}}$

Serum Glutamate Pyruvate Transaminase (Alanine Transaminase) activity was determined following the method of Reitman and Frankel (1957).

Pippeted out 1 ml of buffered substrate into two test tubes labeled as Test and control. Added 0.2 ml of serum to test and incubated the tubes for 30 min. at 37°C. After incubation, 0.2 ml of serum was added to the control tube and 1 ml of 2,4-DNPH reagent was added to both the tubes and kept at room temperature for 20 min. The reaction was stopped by the addition of 10 ml of 0. 4 N NaOH, vortexed and kept at room temperature for 5 min.

The absorbance was measured at 520 nm in a spectrophotometer and the results expressed as enzyme units /ml.

Serum Glutamate Oxaloacetate Transaminase (Aspartate Transaminase was determined following the method of Reitman and Frankel (1957).

Pippeted out 1 ml of buffered substrate into two test labeled as Test and control. Added 0.2 ml of serum to test and incubated the tubes for 60 min. at 37°C. After incubation, 0.2 ml of serum was added to the control tube. 1 ml of 2,4-DNPH reagent was added to both the tubes and kept at room temperature for 20 min. The reaction was stopped by the addition of 10 ml of 0. 4 N NaOH, vortexed and kept at room temperature for 5 min. The absorbance was measured at 520 nm in a spectrophotometer and the results expressed as enzyme units /ml.

Triglycerides were estimated with diagnostic reagent from Qualigens Diagnostics (P.No.72381) manufactured by Sigma diagnostics Ltd (Fossati *et al.,* 1982).

Triglycerides in the sample were hydrolysed by microbial lipases to glycerol and free fatty acids. The glycerol formed is converted to aminoantipyrine and 3,5 Dichloro 2 hydroxy benzene sulfonic acid in a reaction catalyzed by peroxidase. The result of this oxidative coupling is a quinoneimine red coloured dye. The absorbance of this dye in solution is proportional to the concentration of triglycerides in the sample. To three test tubes marked as blank, standard and test, 1 ml of working reagent was added, followed by 10 µl of sample to the test, 10 µl of standard solution to 'standard tube'. The tubes were mixed and incubated for 10 minutes and the absorbance read against blank on a spectrophotometer at 520 nm. The results were expressed as mg/100ml.

$$\text{Triglyceride concentration} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

Lactate dehydrogenase (LDH) was determined with diagnostic reagent from Qualigens Diagnostics (P.No.72351) manufactured by Sigma diagnostics Ltd.

LDH catalyses the reversible oxidation of lactate to pyruvate. Reagents involved are a substrate, buffer and co-enzyme reagent. Working solution was prepared by adding 0.1 ml of substrate to 1 ml of reconstituted co-enzyme reagent .To the test, 1 ml of reagent and 30 µl of serum were added, mixed by inversion, incubated for a minute and initial absorbance was noted after 60 sec and four readings were taken at intervals of 30 seconds at 340 nm. The change in absorbance/ minute was determined from the linear part of the assay.

LDH activity in serum (IU/L) = ? A/min X F

Where F= 5520 (which is calculated on the basis of millimolar absorption of NADH at 340 nm)

3.9. Statistical analysis

Data were presented as mean ± standard deviation and analysed using one and two way analysis of variance (ANOVA). When a significant deviation was found, the mean values were tested for significance ($P<0.05$) by Duncan's multiple range test (Duncan, 1955) and Least significant difference (LSD). Statistical analysis was performed using the SPSS 10.0 version for Windows and results were tested for significance at 5% level.

Chapter IV

Results

4. RESULTS

4.1. Aflatoxin B_1 contamination in fish and shrimp feeds

The results of the survey to detect aflatoxin contamination in fish and shrimp feeds and ingredients from farms, located in Vypeen and Narakkal, Ernakulam District, Kerala are presented in Table 4.1. Feeds obtained from farms had AFB_1 residues within the permissible limits of 20 ppb. The feeds that were obtained from farms and stored for more than six months in the laboratory in plastic containers had visible fungal growth and AFB_1 residues of 150 ppb, which was seven fold higher than the permissible levels of 20 ppb. Feed ingredients like groundnut oil cake that was obtained from local market showed traces of AFB_1, while powdered groundnut oilcake stored for six months in the laboratory in plastic containers had 250 ppb of aflatoxin residue.

Table 4.1. AFB_1 levels recorded in different feeds and feed ingredients

Ingredients	Source and type	AFB_1 levels
Groundnut oilcake	Obtained locally and powdered in laboratory	26 ppb
Groundnut oilcake	Powdered and Stored for 6 months in plastic containers	250 ppb
Shrimp feed	Stored, fungal growth present	150 ppb
Fish feed	Fresh, from farm	18 ppb
Soyabean meal	Obtained from lab	15 ppb
Shrimp feeds	Fresh, from farms	0.0002 ppb

4.2. Growth and feed performance in *P. monodon* postlarvae fed different doses of AFB_1

Three experiments were conducted in *P. monodon* postlarvae to determine the effect of AFB_1 on growth and feed performances.

A. The first experiment conducted in *P. monodon* postlarvae fed AFB_1 incorporated diets for 40 days revealed that the weight gain and survival rates (table 4.2) were significantly ($P<0.05$) higher in the control group than the four treatment groups. Shrimps in the control group and 20 ppb AFB_1 dosed groups showed normal feeding behaviour and morphological features till the end of the experiment. In the control and 20 ppb AFB_1 groups the mean weight gain was 0.498 g and 0.442g, which decreased to 0.253 g in the 50 ppb AFB_1 group. The mean weight gain showed a sharp decline in 2500 ppb (0.094 g) and in 5000 ppb (0.046 g). DMRT on weight gain revealed three homogenous sets of control and 20 ppb group, second set of 50 ppb alone and third grouping of 2500 ppb and 5000 ppb together. Survival rate dropped to 21.6% in 5000 ppb group compared to the rates of 97% and 95% in the control and 20 ppb groups, while in the 50 ppb group the survival rate was 91%.

Table 4.2. Weight gain and survival of *Penaeus monodon* postlarvae fed diets containing selected levels of AFB_1

AFB_1 doses	Weight gain	Survival %
Control	0.498 ± 0.106^a	97.03 ± 1.0^a
20 ppb	0.442 ± 0.097^a	95.26 ± 0.92^a
50 ppb	0.253 ± 0.141^b	91.16 ± 1.25^b
2500 ppb	0.094 ± 0.029^c	51.46 ± 2.83^c
5000 ppb	0.046 ± 0.01^c	21.66 ± 1.52^d

The initial weight of postlarvae was 0.06 ± 0.013g.
Values having a different superscript in the same column are statistically significant.

Histological observations of the different groups of shrimps showed normal structure of the hepatopancreas and cephalothorax region in the control and 20 ppb groups. In 50 ppb group, the hepatopancreas showed only mild change in structure of tubules. In 2500 ppb AFB_1 group, the hepatopancreas had severe necrosis and degeneration of tubules. In 5000 ppb AFB_1 dosed group, the entire tubules of the hepatopancreas were sloughed off and fibrosis was evident in the entire focal area.

Based on the results of this experiment, the doses for other experiments were selected.

B. The effects of the different doses of AFB_1 on weight gain, specific growth rate, feed conversion and survival rate recorded in the second experiment are presented in the table 4.3. There was highly significant effect ($P<0.05$) of the different doses on weight gain, specific growth rate, feed conversion ratio and survival rate. No significant variation was observed between the treatments. Hence another experiment with 3 doses of AFB_1 incorporating other parameters was conducted.

Table 4.3. Response parameters in the second experiment on growth performance in *P. monodon* postlarvae fed AFB_1 diets

Doses of AFB_1	Weight gain %	Specific growth rate	Apparent Feed conversion ratio	Survival%
0 ppb	11.92 ± 0.23^{a}	5.68 ± 0.03^{a}	3.51 ± 0.35^{a}	96.33 ± 1.52^{a}
50 ppb	9.99 ± 1.14^{b}	5.32 ± 0.23^{b}	4.76 ± 0.51^{b}	91.33 ± 1.52^{a}
250 ppb	9.37 ± 0.72^{b}	5.19 ± 0.15^{b}	5.29 ± 0.06^{bc}	84.6 ± 2.08^{b}
500 ppb	8.59 ± 0.78^{c}	5.01 ± 0.17^{bc}	5.86 ± 0.30^{c}	81.6 ± 3.78^{b}
750 ppb	8.67 ± 0.26^{bc}	5.04 ± 0.06^{bc}	6.53 ± 0.304^{d}	80.3 ± 5.03^{b}
1000 ppb	7.45 ± 0.49^{c}	4.74 ± 0.13^{c}	7.54 ± 0.16^{e}	72.3 ± 3.21^{c}
2000 ppb	5.09 ± 0.96^{d}	3.99 ± 0.35^{d}	9.7 ± 0.45^{f}	61.6 ± 4.04^{d}

The initial weight of postlarvae was 0.05 ± 0.001g.
Values having a different superscript in the same column are statistically significant

C. After 45 days in the third experiment, the control group exhibited normal growth, feeding behavior and morphological features. The feed

intake in the control was about 15% of the body weight during the first 15 days, 12% during the next 15 days and 10% during the last 15 days of the experiment. The feed intake was normal in the 50 ppb group during the first 30 days but reduced to 8 % in the last 15 days. In the 500 ppb group, feed intake was normal in the first 15 days, but reduced to 7% after 15 days and 6% of the body weight after 30 days. In the postlarvae dosed with 2000 ppb AFB_1, the feed intake was normal during the first week and decreased to 5% during the last 15 days in the surviving shrimps. There were no gross morphological changes in appearance of the shrimps. Less feed intake and reduction in growth was evident in 500 ppb and 2000 ppb groups.

4.2.C.1. Growth responses

The effect of different levels of AFB_1 on weight gain, specific growth rate, apparent FCR, protein efficiency ratio, net protein utilization, feed digestibility, protein digestibility and survival rate of *P. monodon* postlarvae are represented in the table 4.4.

Weight gain

The percentage weight gain was 7.86 in control, and 2.47 % in highest toxin dosed group (2000 ppb). Weight gain was highly significant among the treatment groups (Table 4.5). Post-hoc comparisons showed that weight gain in the 2000 ppb group was significantly lower ($P<0.05$) than rest of the groups.

Table 4.4. Response of postlarvae of *P. monodon* given different doses of AFB_1

	Doses of Aflatoxin B_1			
Response parameters	**0 ppb**	**50 ppb**	**500 ppb**	**2000 ppb**
Initial wt. (g)	0.137± .039	0.113± .004	0.107± .008	0.108± .011
Final wt. (g)	1.24 ± .483	0.626 ± .08	0.556 ± .038	0.38 ± .087
Weight gain	1.106± .443[a]	0.519 ± .076[ab]	0.448 ± .035[ab]	0.271± .076[ab]
Weight gain %	7.86 ± .85[a]	4.6 ± .862[ab]	4.168 ± .42[ab]	2.477± .469[b]
Specific growth rate %	4.84 ± .362[a]	3.78 ± .127[ab]	3.64± .069[ab]	2.75 ± .23[b]
Apparent FCR	3.25± .211	4.91± .36	5.51± 0.184	8.67± .309
Protein efficiency ratio	0.712± .085[a]	0.496±.103[ab]	0.423± .022[ab]	0.281± .05[b]
Net protein utilization	0.186 ±.07[a]	.068±.011[ab]	.048±.003[ab]	.025±.006[ab]
Feed digestibility %	77.24 ±.855[a]	71.69± 1.087[ab]	69.53 ±2.00[ab]	61.19± .35[b]
Protein digestibility %	89.27 ±.443[a]	81.19 ±.707[ab]	80.01± 1.26[ab]	67.34 ±1.34[b]
Survival rate %	98.33± 0.577[a]	94.33 ±1.527[a]	82.133± 2.2[ab]	66.2 ±2.029[ab]

The initial weight of postlarvae was 0.11 ± 0.02g.
Values having a different superscript in the same row are statistically significant

Table 4.5. ANOVA of weight gain in *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	SS	df	Mean Square	F
Between Groups	45.664	3	15.221	32.588**
Within Groups	3.737	8	.467	
Total	49.401	11		

** Significant at (P<0.01)

Specific growth rate

The mean specific growth rate was 4.84 in the control group, and 2.75 in 2000 ppb AFB_1 group. There was nearly 50% reduction in specific growth rate in 2000 ppb AFB_1 group when compared to the control. Significant effect of aflatoxin on specific growth rate was observed (Table 4.6). DMRT results revealed that specific growth rate in control was significantly ($P<0.05$) greater than all other treatments. Analysis between treatments revealed that mean specific growth for control and 2000 ppb was significantly higher than other groups. The specific growth rate of shrimps in 50 ppb and 500 ppb were not significantly different.

Table 4.6. ANOVA of specific growth rate of *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	SS	df	Mean Square	F
Between Groups	6.583	3	2.194	28.820**
Within Groups	0.609	8	7.614E-02	
Total	7.192	11		

** Significant at ($P<0.01$)

Feed conversion ratio (FCR)

The FCR values showed positive correlation ($r = 0.889$**) with increasing concentration of AFB_1. The mean apparent FCR values were 3.25 in the control, while it was 8.67 in 2000 ppb group. Different doses of AFB_1 had a significant ($P<0.05$) effect on feed conversion ratio. LSD tests demonstrated that the mean FCR at 2000 ppb was significantly greater ($P<0.05$) than all other treatment groups (Table 4.7). There was 60% increase in FCR in 2000 ppb group when compared with the control.

Plate 3

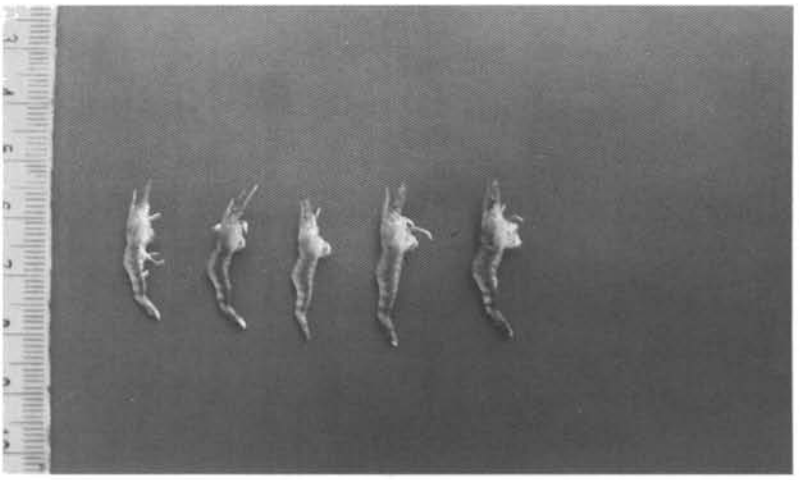


Plate 3a. Initial stage of *P. monodon* postlarvae in the growth performance study

Plate 3b. Final stage of *P. monodon* postlarvae in the control group

Plate 3c. Final stage of *P. monodon* postlarvae in the group given 2000 ppb aflatoxin B_1

Table 4.7. ANOVA of apparent feed conversion ratio in *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	SS	df	Mean Square	F
Between Groups	46.383	3	15.461	16.173**
Within Groups	7.648	8	0.956	
Total	54.031	11		

** Significant at ($P<0.01$)

Feed and protein digestibility

The mean percentage feed digestibility was 77.24 % in control group; while in the 2000 ppb aflatoxin dosed group it was 61% (Fig. 4.1). The mean protein digestibility values decreased with increasing concentration of AFB_1 (Table 4.8). The mean digestibility values at 2000 ppb were significantly ($P<0.05$) less than those at all lower concentrations of aflatoxin (Table 4.9).

Table 4.8. ANOVA of feed digestibility in *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	SS	df	Mean Square	F
Between Groups	398.943	3	132.981	87.992**
Within Groups	12.090	8	1.511	
Total	411.033	11		

** Significant at ($P<0.01$)

Fig. 4.1. Feed and Protein digestibility in *P. monodon* postlarvae fed different doses of AFB_1

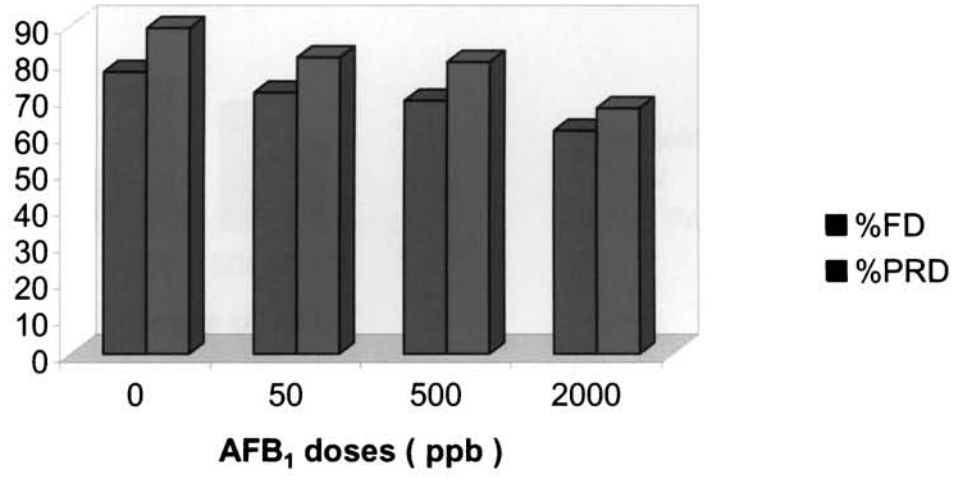


Table 4.9. ANOVA of protein digestibility in *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	SS	df	Mean Square	F
Between Groups	739.208	3	246.403	239.216**
Within Groups	8.240	8	1.030	
Total	747.449	11		

** Significant at ($P<0.01$)

Protein efficiency ratio (PER) and Net Protein utilization (NPU)

Protein efficiency ratio (Fig. 4.2) also varied significantly among AFB_1 concentrations. The mean PER value was significantly ($P<0.05$) less in 2000 ppb group than the other treatments (Table 4.10). Analysis of variance on NPU followed the same trend as in PER and decreased progressively with increase in AFB_1 doses in postlarvae. The 2000 ppb group showed a significant reduction ($P<0.05$) in NPU lower than the values measured in control, 50 ppb and 500 ppb group (Table 4.11).

Fig. 4.2. Protein efficiency ratio and Net protein utilization in *P. monodon* postlarvae given different doses of AFB_1

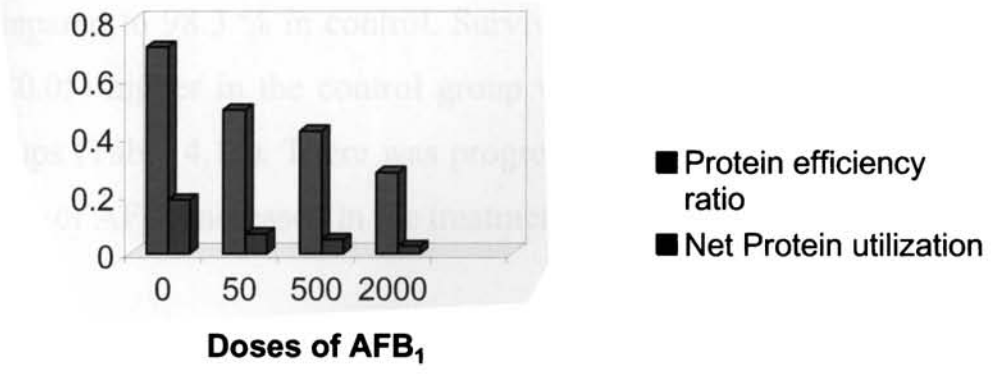


Table 4.10. ANOVA of protein efficiency ratio in *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	.290	3	9.658E-02	18.265**
Within Groups	4.230E-02	8	5.288E-03	
Total	.332	11		

** Significant at (P<0.01)

Table 4.11. ANOVA of net protein utilization in *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	4.634E-02	3	1.545E-02	10.160**
Within Groups	1.216E-02	8	1.520E-03	
Total	5.850E-02	11		

** Significant at (P<0.01)

Survival rate

Survival rate was severely affected in shrimps fed the highest concentration of AFB_1, decreased to 58% after 45 days of treatment when compared to 98.3 % in control. Survival rate was found to be significantly ($P<0.05$) higher in the control group when compared with other treatment groups (Table 4.12). There was progressive decrease in the survival rate as doses of AFB_1 increased in the treatment groups (Fig. 4.3).

Fig. 4.3. Survival rate in *P. monodon* postlarvae at different doses of AFB_1

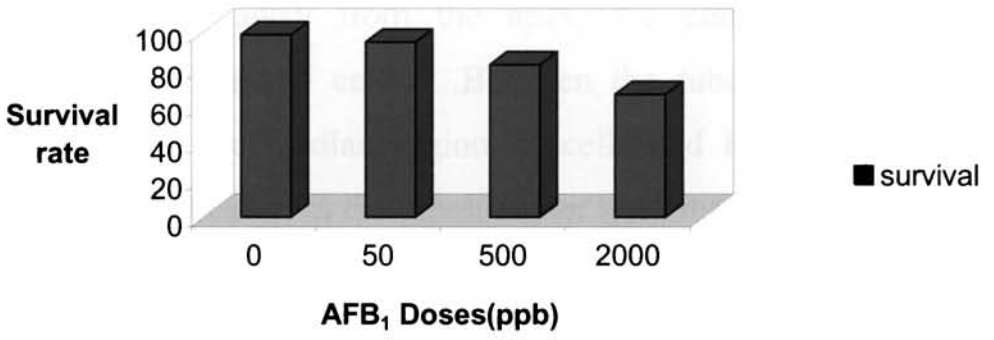


Table 4.12. ANOVA of survival rate of *P. monodon* postlarvae fed different doses of AFB_1

Source of variation	SS	df	Mean Square	F
Between Groups	1878.890	3	626.297	215.222**
Within Groups	23.280	8	2.910	
Total	1902.170	11		

** Significant at ($P<0.01$)

Histological changes

The cephalothorax of postlarvae from all the experimental groups was observed for morphological and histological changes. In the control, the cephalothorax showed normal architecture of hepatopancreas, lymphoid organ, antennal gland and gills (Plate 4,5).

In the control group, the hepatopancreas was normal with the proximal and the apical region, specific components of stomach and midgut namely the gastric sieve and the lappets (Plate 5a). The lumen contained a granular material and lumen-facing surface of the tubule was covered with a microvillus border. The tubular apex contained undifferentiated embryonic cells (E cells). Proceeding away from the apex, the cells began to differentiate into storage cells (R- cells). Between the tubules, haemal sinuses were observed. In the median region, R cells and F cells were observed. Those F cells farthest from the tubular apex were more basophilic and larger than those nearest the apex. F cell nuclei were larger than those of R cells and typically contained one prominent nuclei. The cytoplasm of R cells characteristically contained numerous nuclei. The proximal region of the tubule contained the large distinctive secretory cells (B cells), each of which contained one large vacuole and a convex luminal surface (Plate 4a-4c).

The hepatopancreas of 50 ppb AFB_1 dosed postlarvae revealed normal B cells and atrophy of R cells (Plate 6a). The lymphoid organ, antennal gland, gills and muscle tissue appeared to be normal. In the 500 ppb group, the hepatopancreas revealed cell elongation, hyperplasia and slight necrosed areas (Plate 6b). In the 2000 ppb group, the hepatopancreas showed completely desquamated tubules (Plate 6c). Muscle tissue was detached with no striations. Necrosis of hepatopancreas and antennal gland was observed.

Plate 4

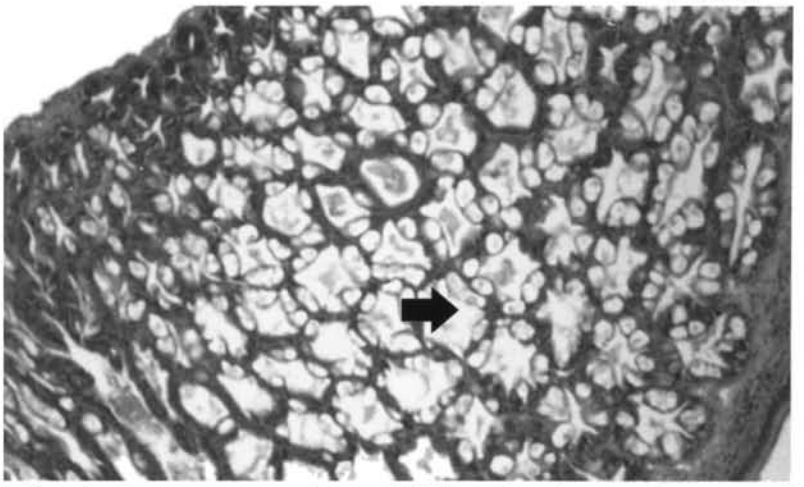


Plate 4a. Hepatopancreas of control shrimp. Note the normal structure of tubules, lumen and cells. H&E 100x

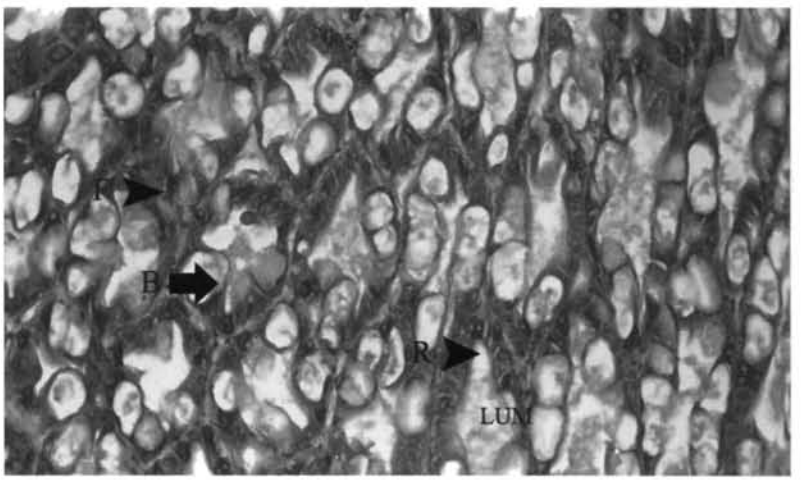


Plate 4b. Section of hepatopancreas of control shrimp showing B, R and F cells, and the lumen of the tubules. H&E 200x

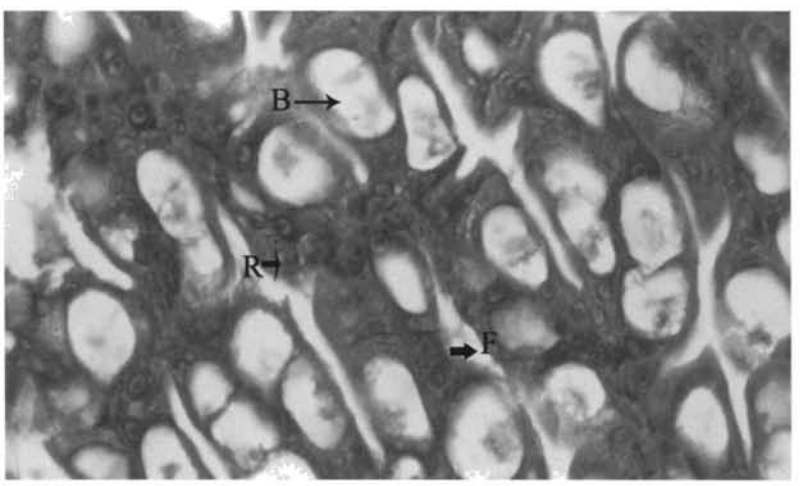


Plate 4c. Enlarged view of the section of the hepatopancreas of control shrimp showing different cells. H&E 400x

Plate 5

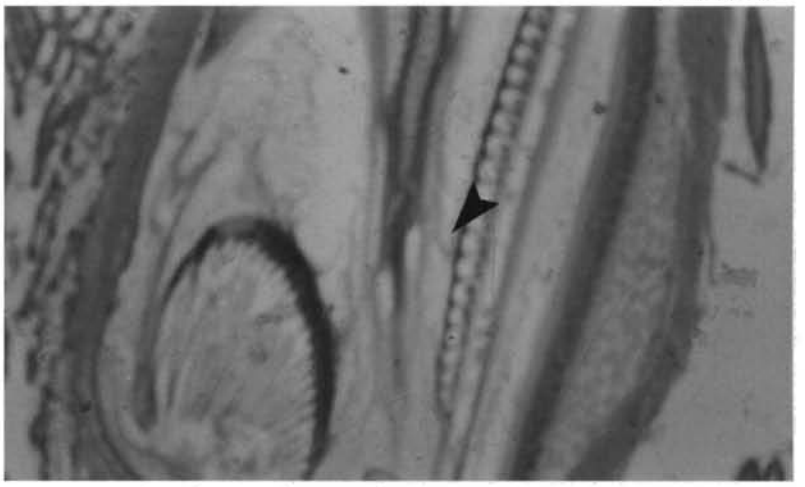


Plate 5a. Section showing the normal structure of the midgut region in the control shrimp. H&E 100x

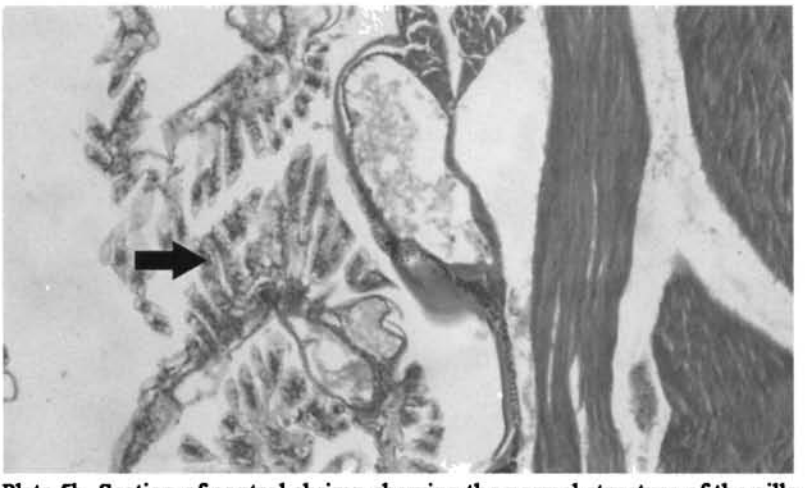


Plate 5b. Section of control shrimp showing the normal structure of the gills and muscles. H&E 100x

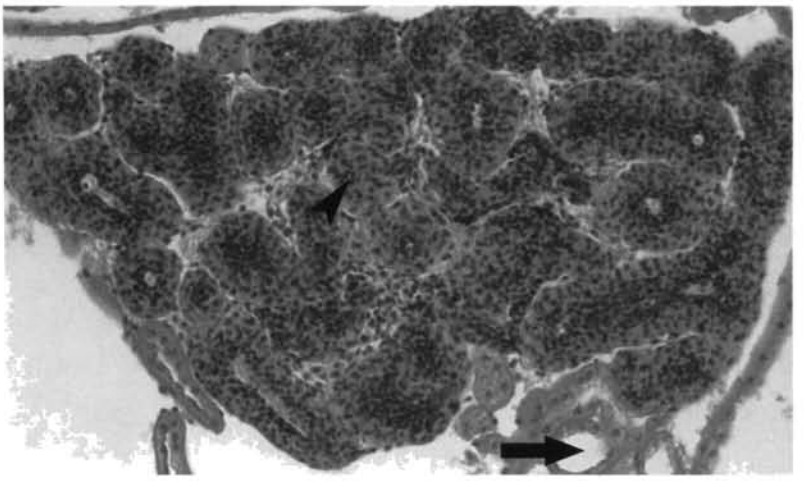


Plate 5c. Section of control shrimp. Note the normal structure of the lymphoid organ (arrow head) and antennal gland (arrow). H&E 200x

Plate 6

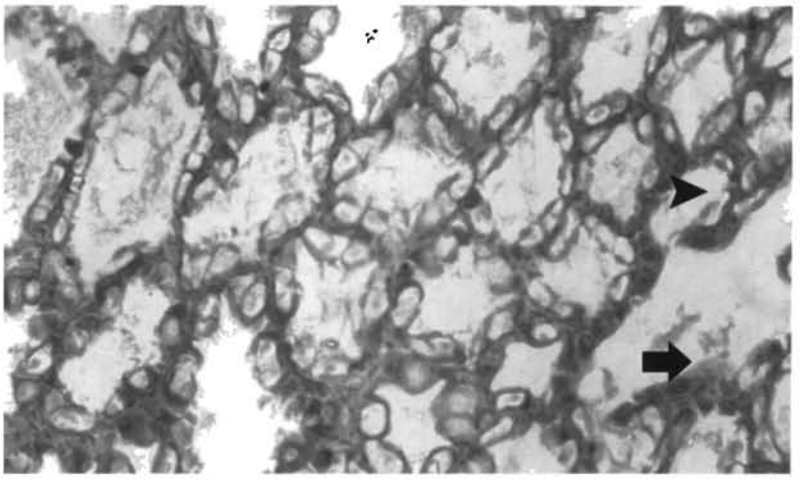


Plate 6a. Section of hepatopancreas of *P. monodon* postlarvae given 50 ppb aflatoxin B_1 in the diet. Note the change in structure of the tubules (arrow head)and loss of tubules (arrow). H&E 200x

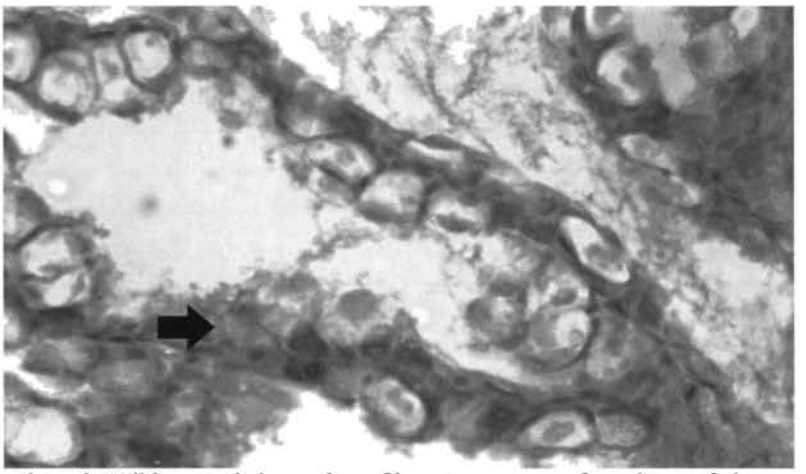


Plate 6b. Mild necrosis in section of hepatopancreas of postlarvae fed 500 ppb aflatoxin B_1 in the diet. H&E 400x

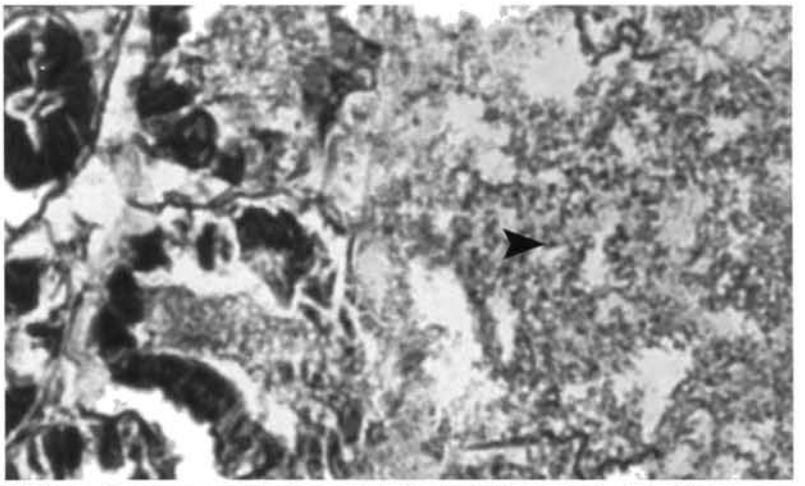


Plate 6c. Severe necrosis observed in the hepatopancreas of postlarvae given 2000 ppb aflatoxin B_1. H&E 400x

4.3. Interaction of Aflatoxin B_1 with sub-lethal levels of copper/ cadmium in *P. monodon* postlarvae

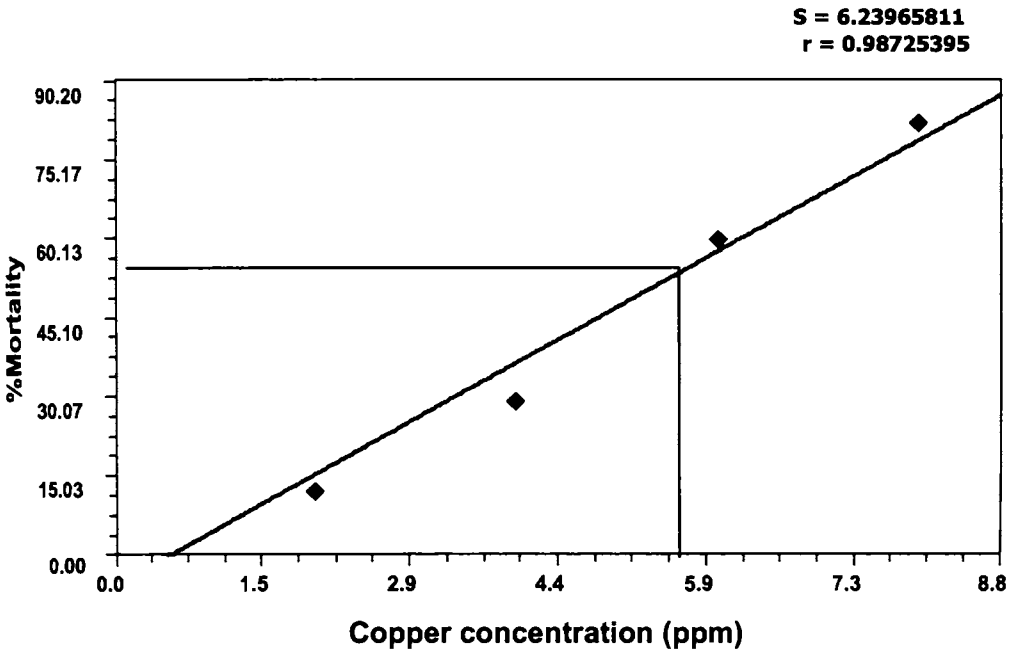
4.3.1.a. Determination of 96 hr LC_{50} of copper

The percentage mortality of *P. monodon* postlarvae to different concentration of copper are presented in the table 4.13. From the best-fit plots (Fig. 4.4.), the LC_{50} at 96 hours of copper was determined and the sub-lethal dose was selected for study.

Table 4.13. Percentage mortality of *P. monodon* postlarvae at different concentrations of copper

LC $_{50}$ of copper	
Conc. ppm	Death%
0	0
2	12 ±2
4	29±1
6	60±3
8	82±1

Fig. 4.4. Response curve of *P. monodon* postlarvae to different concentration of copper



Linear Fit: y=a+bx, Coefficient Data: a = -5.8, b = 10.6
LC_{50} of copper = 5.26 ppm
1/10 of LC_{50} for copper (0.526 ppm) was taken as sub-lethal dose for study

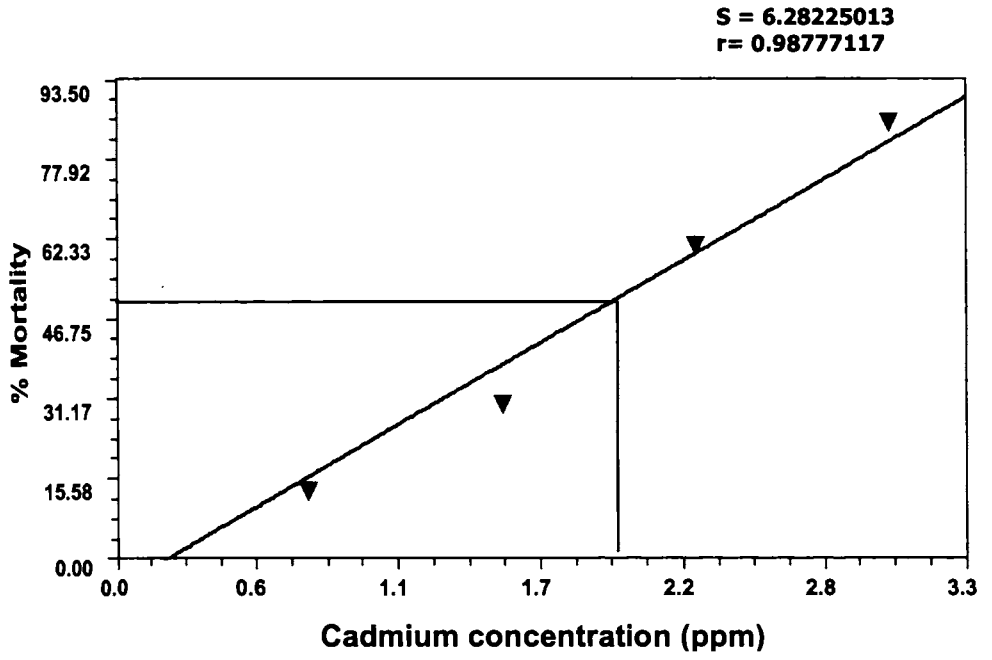
4.3.1.b. Determination of 96 hr LC_{50} of cadmium

The percentage mortality of *P. monodon* postlarvae to different concentration of cadmium are presented in the table 4.14. From the best-fit plots (Fig. 4.5.), the LC_{50} at 96 hours of cadmium was determined and the sub-lethal dose was selected for study.

Table 4.14. Percentage mortality of *P. monodon* postlarvae of to different doses of cadmium

LC_{50} of cadmium	
Conc. ppm	Death%
0	0
0.75	13±3
1.5	30±1
2.25	61±2
3	85±3

Fig. 4.5. Response curve of *P. monodon* postlarvae to different concentration of cadmium



Linear Fit: y=a+bx, Coefficient Data: a = -5.8, b= 29.066667
LC_{50} of cadmium=1.92 ppm
The sub-lethal level (1/10 of LC_{50}), hence 0.192 ppm was taken for study.

4.3.2. Growth responses

The results of the growth responses observed in the interaction study are presented in table 4.15. In the control group, the postlarvae showed normal behaviour, survival and growth during the experimental duration of 40 days. The second group (500 ppb AFB_1) revealed reduction in growth when compared to the control group. The third group (copper and 500 ppb aflatoxin B_1) survived only up to 25 days of the experiment and the growth data presented for copper + toxin are as observed during 24 days duration. The survival rate in this group was 34% after 15 days of experiment (Fig.4.6). The mean increase in weight was 0.305 and percentage weight gain was 0.645. The specific growth rate and apparent feed conversion ratio showed a mean value of 1.2 and 24.8. The protein efficiency ratio and net protein utilization revealed a mean value of 0.354 and 0.183. The fourth group (cadmium and 500 ppb aflatoxin) revealed 74% survival rate till 40 days of the experiment. Hence the statistical significance was determined only based on control, aflatoxin group and cadmium + toxin group.

Fig.4.6. Survival rate of *P. monodon* postlarvae in interaction study

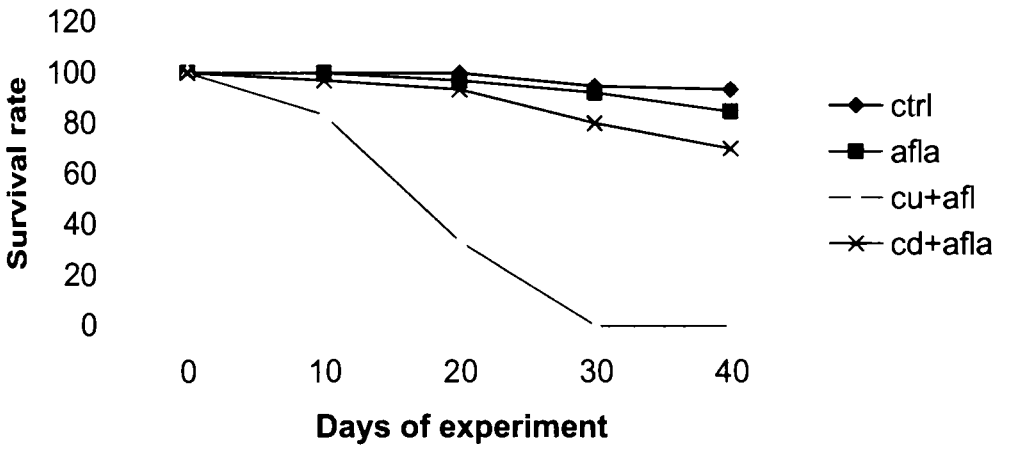


Table 4.15. Growth responses of *P. monodon* postlarvae given 500 ppb aflatoxin in the feed and sub-lethal doses of copper/ cadmium in the rearing medium

Response parameters	Control	AFB_1	Cu+ AFB_1	Cd+AFB_1
Initial wt. g	0.528 ± 0.028	0.503 ± 0.109	0.490 ±0.045	0.548 ±0.03
Final wt. g	4.66 ± 0.50	2.51± 0.33	0.795 ±0.107	2.008 ± 0.53
Weight gain	4.138 ± 0.531	2.01± 0.319	0.305± 0.152	1.459 ±0.513
Weight gain %	7.88 ±1.44	3.98 ± 0.509	0.645± 0.38	2.64 ± 0.877
Specific growth rate	5.44 ± 0.402	4.007± 0.25	1.20 ±0.567	3.186 ± 0.584
Apparent FCR	3.53 ± 0.548	6.22 ± 0.55	24.8 ± 8.88	7.988± 4.32
Protein efficiency ratio	0.66 ± 0.106	0.376± .034	0.101± 0.03	0.354± 0.18
Net protein utilization	0.747± 0.08	0.296± .044	0.056± 0.01	0.183 ± 0.05
Survival %	93.6 ± 0.79	84.66 ± 5.03	34.33 ± 2.08	74.3 ± 2.5

The initial weight of postlarvae was 0.55 ± 0.04g.

Weight gain

Weight gain was significantly ($P<0.05$) higher in control than AFB_1 group and cadmium + toxin group (Table 4.16). In the control group and aflatoxin group, the mean weight increase was 4.138g and 2.01 g respectively, while in cadmium + toxin group, the mean weight increase was 1.459 g only.

Table 4.16. ANOVA of weight gain in postlarvae in the AFB_1- heavy metal interaction study

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	44.558	2	22.279	21.422**
Within Groups	6.240	6	1.040	
Total	50.798	8		

** Significant at ($P<0.01$)

Specific growth rate

The mean specific growth rate was significantly ($P<0.05$) less in cadmium + toxin group than other two groups (Table 4.17). There was 40 % reduction in SGR in cadmium + toxin group when compared with the control.

Table 4. 17. ANOVA of specific growth rate in postlarvae in the AFB_1- heavy metal interaction study

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	7.808	2	3.904	20.611**
Within Groups	1.136	6	0.189	
Total	8.944	8		

** Significant at ($P<0.01$)

Apparent feed conversion ratio

Apparent feed conversion ratio between the treatments was not significant (Table 4.18). There was more than 50% increase in AFCR in cadmium + toxin when compared with the control group.

Table 4.18. ANOVA of apparent feed conversion ratio in postlarvae in the AFB_1- heavy metal interaction study

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	30.695	2	15.348	2.414, NS
Within Groups	38.147	6	6.358	
Total	68.842	8		

NS- Not significant

Protein efficiency ratio (PER) and Net protein utilization (NPU)

The protein efficiency ratio was significant ($P<0.05$) among the groups (Table 4.19). The mean PER values were 0.66 in control, 0.376 in AFB_1 group and 0.354 in cadmium group. ANOVA of net protein utilization (Table 4.20) revealed significantly ($P<0.05$) lower values in cadmium +toxin group than the control, the mean NPU values were 0.747 in control, 0.296 in AFB_1 group and 0.183 in cadmium + toxin group

Table 4.19. ANOVA of Protein efficiency ratio in *P. monodon* postlarvae in the AFB_1- heavy metal interaction study

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	.175	2	8.755E-02	5.761*
Within Groups	9.118E-02	6	1.520E-02	
Total	0.266	8		

* Significant at ($P<0.01$)

Table 4.20. ANOVA of net protein utilization in *P. monodon* postlarvae in the AFB_1- heavy metal interaction study

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	.535	2	.268	63.181**
Within Groups	2.541E-02	6	4.235E-03	
Total	.561	8		

** Significant at ($P<0.01$)

Histological changes

Normal structure of the cephalothorax region was observed in the control group (Plate 7a). In the third group (copper + AFB_1), the hepatopancreas revealed severe necrosis and rounding of cells (Plate 7b).

Plate 7

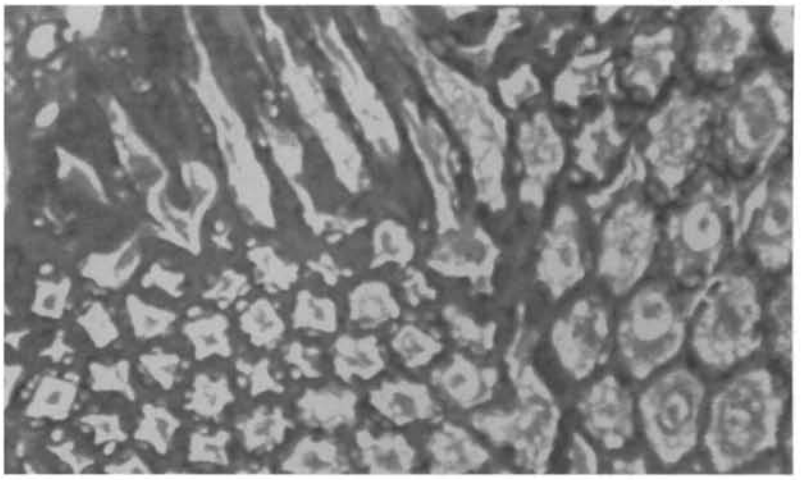


Plate 7a. Hepatopancreas of *P. monodon* postlarvae in the control shrimp in the AFB_1- heavy metal in the interaction study. H&E 200x

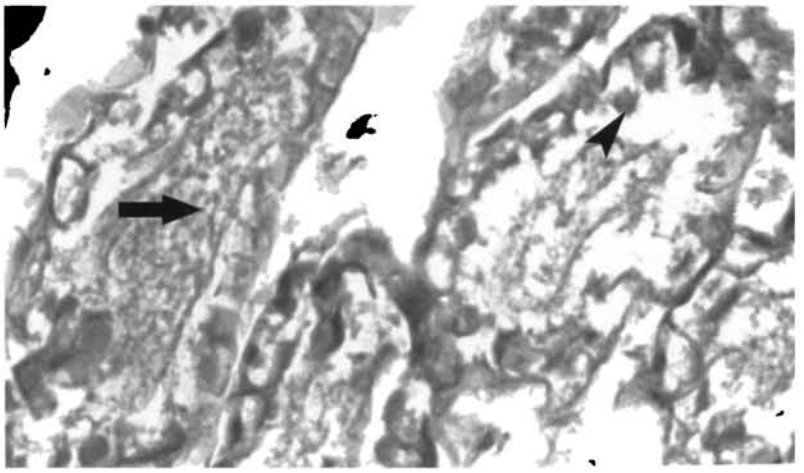


Plate 7b. Section of hepatopancreas of shrimp treated with copper and aflatoxin B_1 revealing severe necrosis (arrow) and rounding of cells (arrow head). H&E 400x

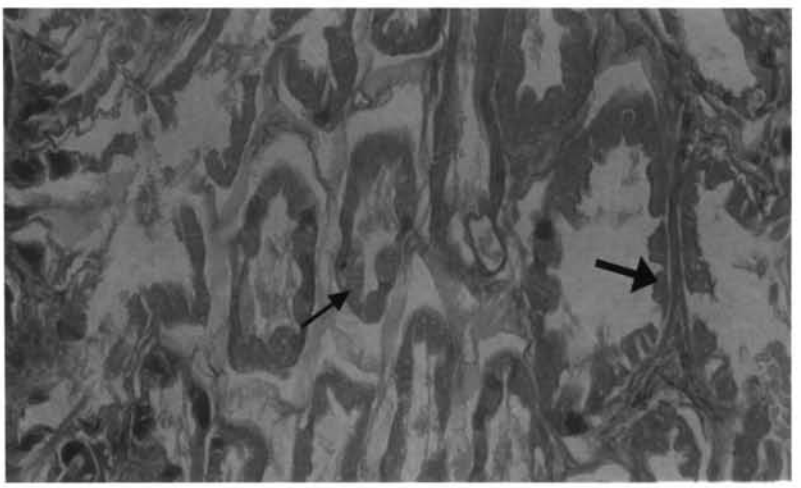


Plate 7c. Section of hepatopancreas of shrimp treated with cadmium and aflatoxin B_1 showing desquamation of cells (arrow) and loss of structure of tubules (small arrow). H&E 200x

While in the fourth group, (cadmium + AFB_1), the changes observed in the hepatopancreas were slight fibrosis, desquamation and loss of structure (Plate 7c).

4.4. Pathological and immunological changes in *P. monodon* sub- adults fed different doses of AFB_1

Control group

In the control group, the shrimps exhibited normal growth, survival, behaviour and feeding (Plate 8a).

Gross Pathology in treatment groups

Sub-adult shrimps fed with 1000 ppb (1ppm) and 2000 ppb (2 ppm) AFB_1 in the diets exhibited slow growth and high mortality at the end of 4 weeks of experiment. Poor feed intake was observed in 500 ppb, 1000 ppb and 2000 ppb AFB_1 treated groups at 4 weeks. In 1000 ppb toxin fed group, tail rot was noticed in about 50% of the surviving shrimps (Plate 8b). Reddish discoloration was observed in the cephalothorax, abdomen and also in the faecal matter in 500 ppb, 1000 ppb and 2000 ppb toxin fed groups (Plate 8c). Hepatopancreas of shrimps in the 2000 ppb treated group turned first red and later pale yellow and was reduced to half of its size than the control group (Plate 9b). Moribund shrimps showed erratic movements and soft shell in 1000 and 2000 ppb group.

Survival rate

In the control group, the survival rate (Table 4.21) was high with mean value of 97.4 %, while the survivability of shrimps in the treatment groups given 500 ppb, 1000 ppb and 2000 ppb aflatoxin B_1 were affected after 4 weeks. The survival rate in higher doses of aflatoxin (1000 and 2000 ppb) fed group were 66% and 56.4%.

Plate 8

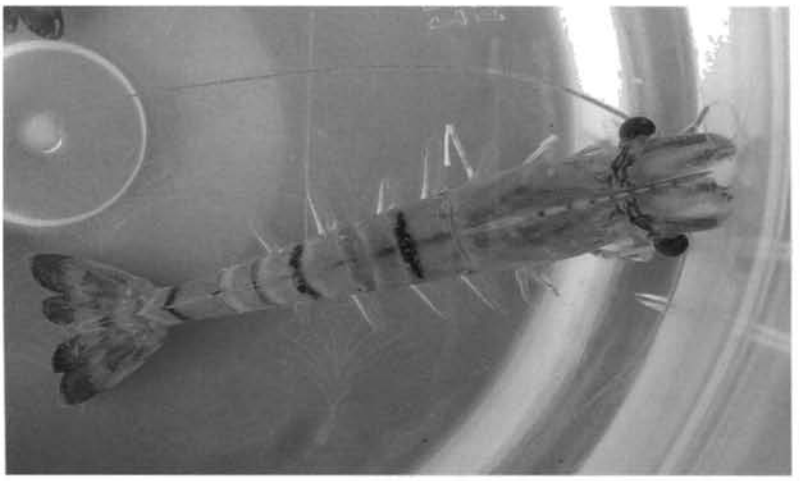


Plate 8a. *P. monodon* in the control shrimp

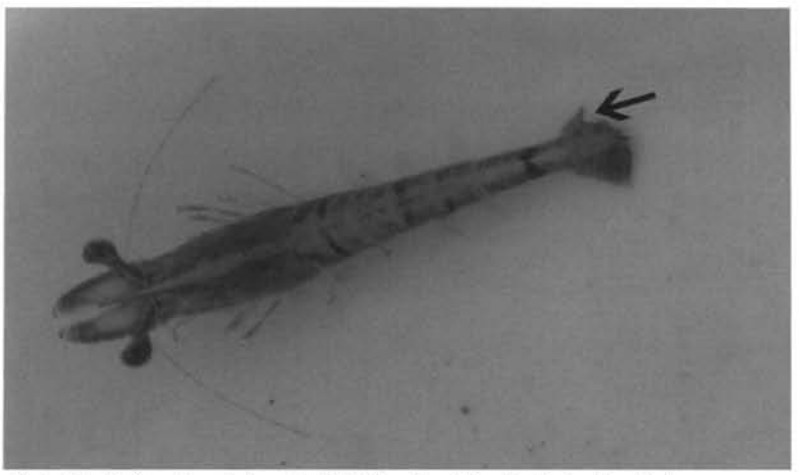


Plate 8b. Tail rot in shrimp fed 1000 ppb aflatoxin B_1 in the diet

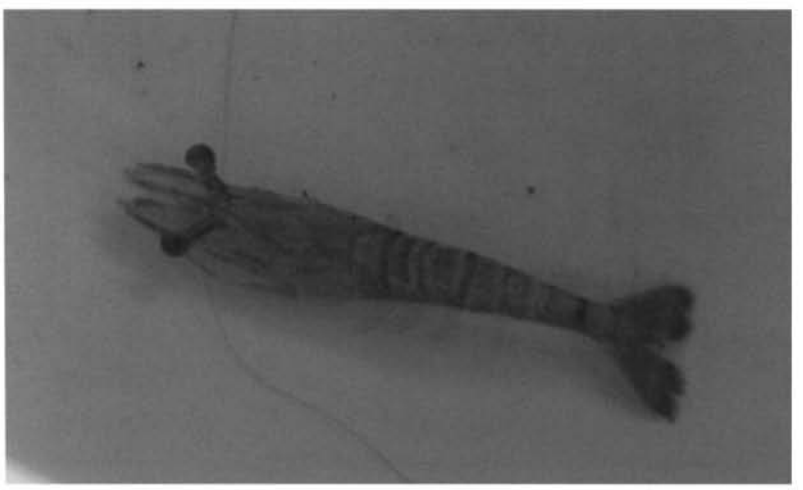


Plate 8c. Reddish discolouration observed in shrimps fed 2000 ppb aflatoxin B_1 in the diet

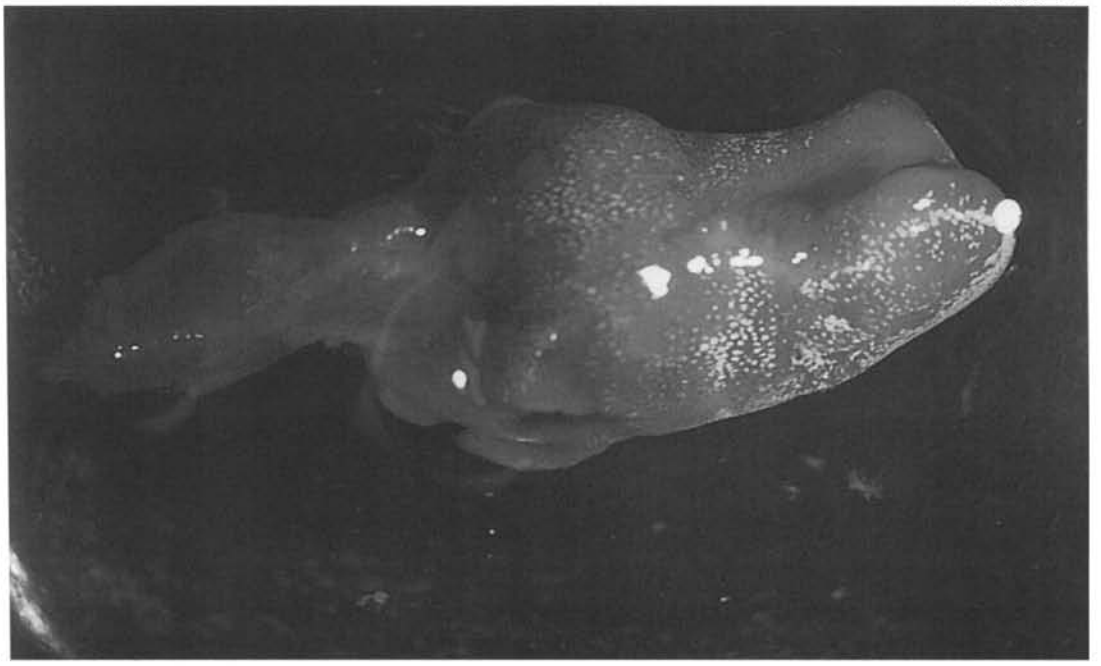


Plate 9a. Hepatopancreas of control shrimp showing normal size and colour

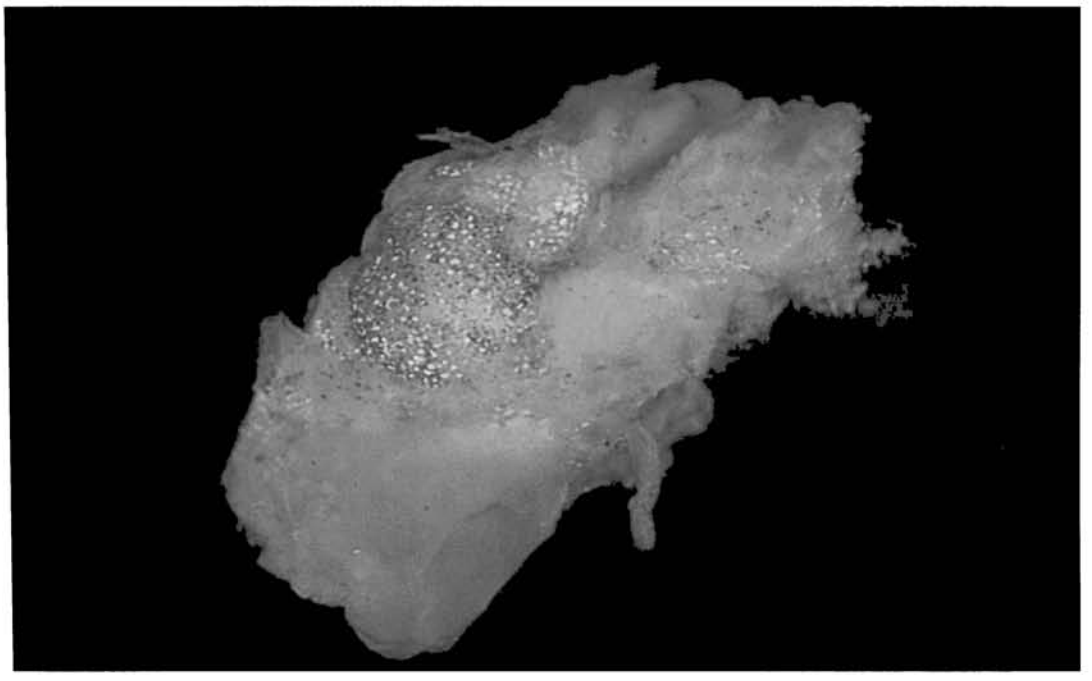


Plate 9b. Hepatopancreas of shrimp given 2000 ppb aflatoxin B_1 showing reduced size, altered shape and pale discolouration

Table 4.21. Survival of shrimps at different doses of AFB_1

Doses of AFB_1	Survival %
0 ppb	97.4 ± 4.4^a
50 ppb	92.3 ± 7.6^b
100 ppb	84.6 ± 7.6^b
150 ppb	82.05 ± 8.8^b
500 ppb	79.4 ± 4.4^b
1000 ppb	66.6 ± 4.4^c
2000 ppb	56.4 ± 8.8^c

Values having a different superscript in the same column are statistically significant

Total haemocyte count (THC)

The total haemocyte count (Table 4.22) showed gradual increase with increasing concentration of AFB_1 after 4 weeks of treatment. After 8 weeks, there was gradual decrease in the number of circulating haemocytes (Fig. 4.7). The mean total count was 1237 x 10^4 cells/ml and 2169 x 10^4cells/ml in the control at 4 and 8 weeks while in the highest toxin fed group (2000 ppb), the THC was 2588 x 10^4 cells/ml and 468.3 x 10^4 cells/ml at 4 and 8 weeks. The effect of different doses on total haemocyte count revealed high level of significance ($P<0.05$) at 4 and 8 weeks (Table 4.23). The total haemocyte count was significantly ($P<0.05$) lower in 150 ppb and 2000 ppb treatment group. The LSD results revealed high level of variance between 150 ppb and 1000 ppb treatments. Correlation between dose and count at 4 weeks demonstrated that there was high positive correlation ($r = 0.800^{**}$), while they were negatively correlated at 8 weeks ($r = -0.882^{**}$) (Plate 10a-10b).

Table 4.22. Effect of AFB_1 on total haemocyte count at 4 and 8 weeks

Doses of AFB_1 (ppb)	THC x 10^4 cells/ml	
	4 weeks	8 weeks
0	2355 ± 65.2	2169.87±258
50	1615± 96.49	1758.75±165
100	1580.25 ±72.4	1246.9 ±128
150	1314.66 ± 353	993.3 ±100
500	1982.5 ± 165	1102.75±203
1000	2512 ± 411	1151 ± 71
2000	2588.3 ± 450	463.3 ± 30

Fig. 4.7. Total haemocyte count at 4 and 8 weeks of AFB_1 treatment

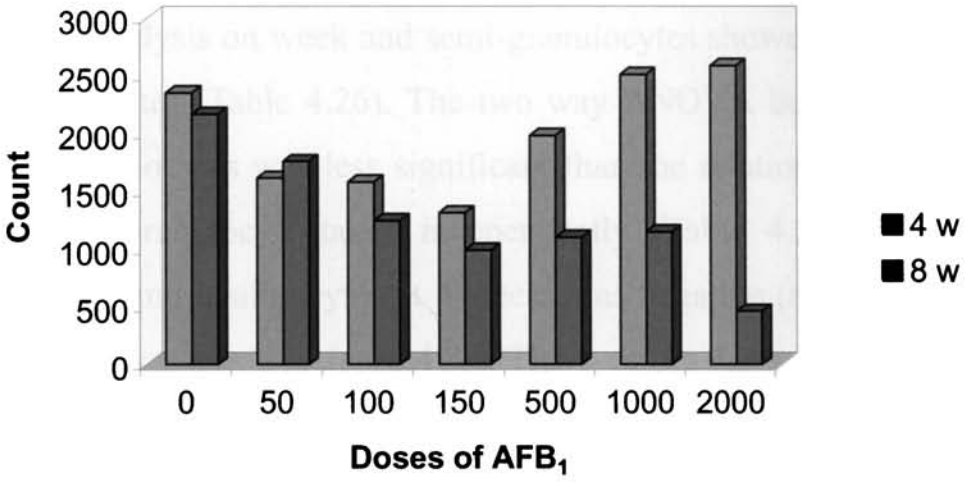


Table 4.23. ANOVA table representing the interactive effect of dose and duration on total haemocyte count

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	65641.941	1	65641.941	1867.503	.000
Dose	729.393	6	121.565	3.459	.009
Week	933.081	1	933.081	26.546	.000
Error	1195.085	34	35.150		

Significant at ($P<0.05$)

Differential haemocyte count (DHC)

DHC (Fig. 4.8) did not vary significantly between the control and treatment groups both after 4 and 8 weeks of experiment (Table 4.24). Hyalinocytes (HC) formed almost 30%, semi granulocytes (SG) almost 60-70% and granulocytes (G) about 10-15 % of the total haemocyte count. Hyalinocytes were almost ovoid cells with blue nucleus and clear cytoplasm. The cells were smaller in size due to small nucleus. Semi-granulocytes were of ovoid to spindle shape with larger nucleus and larger cell size with few granules. While granulocytes, had blue nucleus with large number of acidophilic granules. Analysis of variance of hyalinocytes data was highly significant between weeks (Table 4.25). But the analysis between dose and week and between doses on hyalinocytes was not significant. Analysis on week and semi-granulocytes showed similar results as in hyalinocytes (Table 4.26). The two way ANOVA between dose and week on granulocytes was less significant than the relation between week and dose on granulocyte taken independently (Table 4.27). Correlation between Dose and hyalinocytes at 4 weeks was negative (r =–0.258) while at 8 weeks it was positive (r =0.452). The r-values for semi-granulocytes were –0.361 and –0.405 at 4 and 8 weeks and granulocytes showed –0.302 and 0.035 after 4 and 8 weeks (Plate 10c-10d).

Figure 4.8. Differential haemocyte count at 4 and 8 weeks of AFB_1 exposure

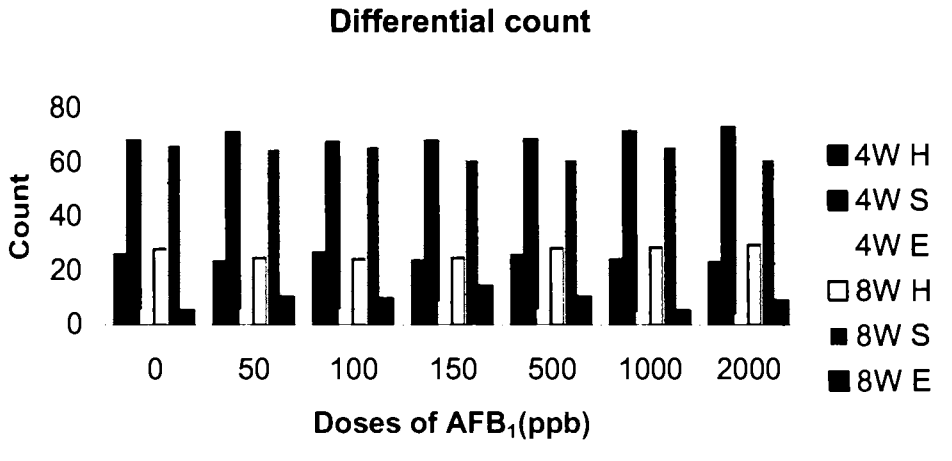


Table 4.24. Differential haemocyte count in *P. monodon* sub-adults at 4 and 8 weeks of AFB_1 exposure

Doses (ppb)	Week	Differential count		
		HC %	SG%	G%
0	4	26 ±2	68.33±3.51	5.66±1.52
	8	28±2	66.66±2.88	5.33±1.52
50	4	23.33±2.51	71.33±2.51	5.33± 0.577
	8	24.66±1.52	65±3	10.33±2.88
100	4	26.66±1.527	67.66±2.51	5.66±1.15
	8	24.33±2.51	66±2	9.66±2.51
150	4	23.66±3.78	68±5.56	8.33±2.08
	8	24.66±1.52	61±2.64	14.33±1.52
500	4	25.66±2.08	68.66±3.05	5.66±2.51
	8	28.33±1.527	61.33±2.51	10.33±2.30
1000	4	24±1.73	71.66±1.52	4.33±1.15
	8	28.66±2.51	66±4	5.33±1.52
2000	4	23±2.64	73.33±1.52	3.66±1.52
	8	29.66±2.51	61.33±2.08	9±2

Table 4.25. ANOVA of hyalinocyte in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Dose	56.619	6	9.437	1.852	.125
Week	54.857	1	54.857	10.766	.003
Dose * week	73.476	6	12.246	2.403	.053
Error	142.667	28	5.095		

NS- Not significant

Table 4.26. ANOVA of semi-granulocytes in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Dose	91.571	6	15.262	1.709	0.156
Week	372.024	1	372.024	41.667	0.000
Dose * week	114.810	6	19.135	2.143	0.080
Error	250.000	28	8.929		

NS-Not significant

Table 4.27. Interaction of doses and duration on granulocytes in *P. monodon*

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	2273.357	1	2273.357	515.294	0.000
Dose	164.476	6	27.413	6.214	0.000
Week	141.167	1	141.167	31.998	0.000
Error	150.000	34	4.412		

Significant at ($P<0.01$)

Phagocytosis

Percentage phagocytosis was found to increase in the treatment groups when analysed at the end of 4 weeks in a dose dependent manner, while decreased phagocytic ratio was observed after 8 weeks of exposure to AFB_1 (Table 4.28). The effect of AFB_1 doses on phagocytosis at 4 and 8 weeks was highly significant ($P<0.05$) but the individual effect of different doses on phagocytosis was not significant (Table 4.29) (Plate 10e -10f).

Table 4.28. Phagocytosis in *P. monodon* sub-adults at 4 and 8 weeks of AFB_1 exposure

Doses of AFB_1 (ppb)	Phagocytic ratio	
	4 weeks	8 weeks
0	60.83±1.75	61.5 ±0.5
50	59.586±1.12	59.75 ±1.56
100	60.83±2.46	59.15± 1.15
150	63.43 ± 0.81	57.43 ±1.20
500	62.66± 2.08	57.91± 1.9
1000	62.83 ±2.36	56.7± 3.3
2000	65.63± .85	52.8 ±0.39

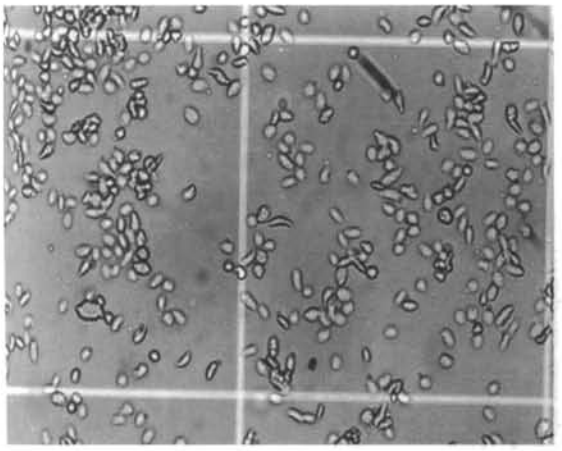


Plate 10a. Total haemocyte count in control group

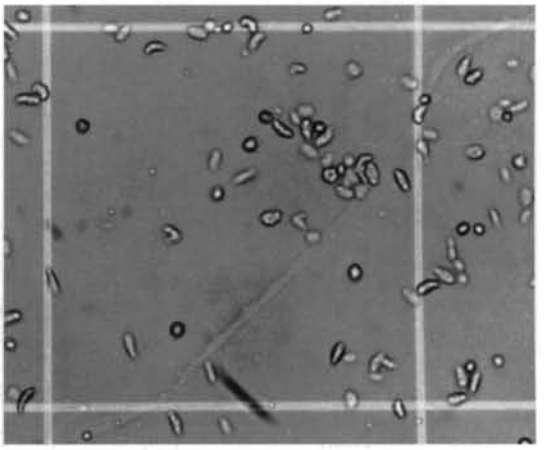


Plate 10b. Total haemocyte count in 2000 ppb aflatoxin B_1 fed group

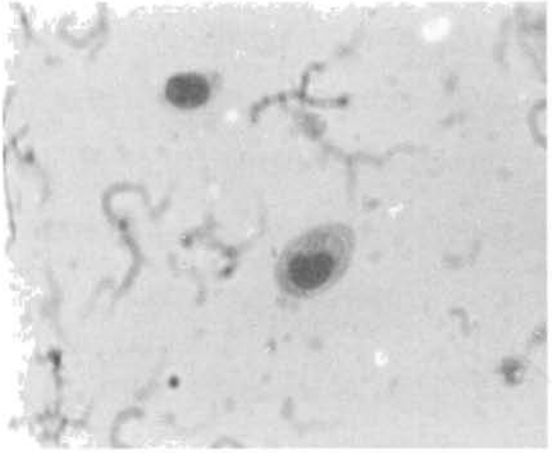


Plate 10c. Differential haemocyte count showing granulocyte and hyalinocyte in the control shrimp

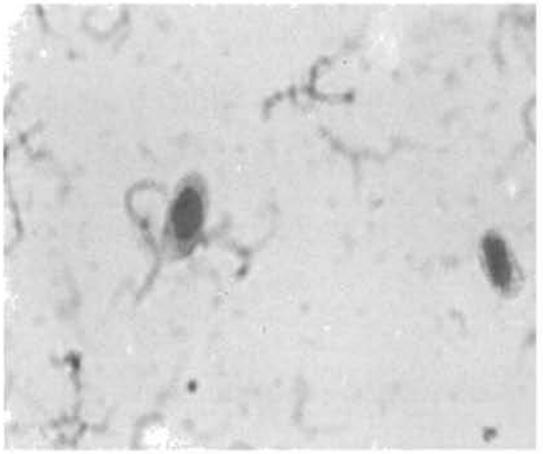


Plate 10d. Differential haemocyte count revealing semi-granulocytes in the control shrimp

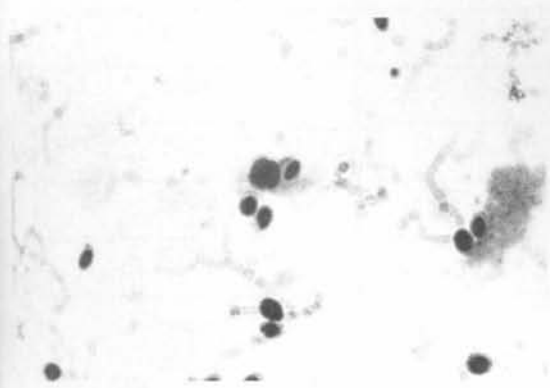


Plate 10e. Yeast particles attached to haemocytes in the control shrimp

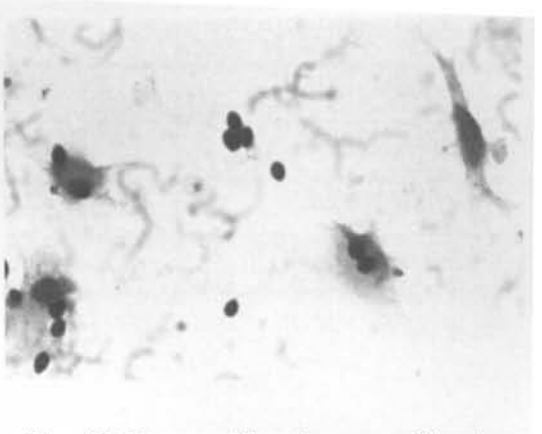


Plate 10f. Yeast particles phagocytosed by the haemocytes in the control shrimp

Table 4.29. ANOVA of percentage phagocytosis in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	152537.344	1	152537.344	19518.314	0.000
Dose	12.324	6	2.054	0.263	0.950
Week	234.679	1	234.679	30.029	0.000
Error	265.713	34	7.815		

NS- Not significant

Phenoloxidase (PO)

There was highly significant ($P<0.05$) effect of different doses of AFB_1 on phenoloxidase activity (Table 4.30) at 4 and 8 weeks of experiment. Phenoloxidase activity in the control group was significantly ($P<0.05$) higher than other treatment groups (Table 4.31). DMRT results showed high variation between control and 1000 ppb AFB_1 fed group. Phenoloxidase and AFB_1 doses exhibited positive correlation at 4 weeks (r= 0.857**) and negative correlation at 8 weeks (r= -0.842**).

Table 4.30. Phenoloxidase activity in *P. monodon* sub-adults at 4 and 8 weeks of AFB_1 exposure

Doses of AFB_1 (ppb)	Phenoloxidase activity	
	4 weeks	8 weeks
0	0.384 ±. 005	0.309 ±0.02
50	0.651± 0.021	0.301± 0.04
100	1.93 ± 0 .06	0.304± 0.02
150	1.86 ± 0.305	0.231± 0.02
500	3.25 ± 0.375	0.245± 0.03
1000	3.5 ± 0.05	0.189± 0.03
2000	3.42 ± 0.446	0.175 ±0 .03

Table 4.31. ANOVA of phenoloxidase activity in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	60.250	1	60.250	118.177	0.000
Dose	14.176	6	2.363	4.634	0.002
Week	37.662	1	37.662	73.873	0.000
Error	17.334	34	0.510		

Significant at ($P<0.05$) level

Acid phosphatase (ACP)

The mean acid phosphatase levels (Table 4.32) showed a rise at 50 ppb, 150 ppb and 2000 ppb viz, 22.28, 18.87 and 22.81 after 4 and 8 weeks. There was significant ($P<0.01$) effect of different doses of AFB_1 on acid phosphatase levels at 4 and 8 weeks. The acid phosphatase levels were significantly lower in 100 ppb group (Table 4.33) and LSD results showed higher level of significance between 100 ppb and 50 ppb. Negative correlation was observed at 4 weeks ($r = - 0.028$) and positive correlation ($r = 0.347$) at 8 weeks.

Table 4.32. The acid phosphatase levels at 4 and 8 weeks in *P. monodon* sub-adults fed AFB_1 diets

Doses of AFB_1 (ppb)	ACP (K.A. units)	
	4 weeks	8 weeks
0	13.96 ±0.45	15.56 ±1.0
50	13.77± 1.46	22.28 ±2.65
100	10.78± 1.06	13.3± .88
150	14.13 ±1.76	18.87± 1.51
500	19.5 ±4.154	16.33 ±1.0
1000	11.08± 1.31	18.38± .72
2000	12.44 ±1.27	22.81± 2.5

Table 4.33. ANOVA of acid phosphate activity in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	10681.396	1	10681.396	1267.081	0.000
Dose	176.036	6	29.339	3.480	0.009
Week	218.014	1	218.014	25.862	0.000
Error	286.617	34	8.430		

Significant at ($P<0.05$) level

Alkaline phosphatase (ALP)

The alkaline phosphatase levels at 4 and 8 weeks of AFB_1 exposure in *P. monodon* sub-adults are presented in the Table 4.34 and Fig. 4.9. Two-way analysis between dose and week on alkaline phosphatase levels revealed that they are not statistically significant at ($P<0.05$). But the individual effects of dose and week on alkaline phosphatase were highly significant (Table 4.35). They showed positive correlation after 4 and 8 weeks ($r = 0.812^{**}, 0.914^{**}$).

Table 4.34. Alkaline phosphatase activity in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Doses of AFB_1 (ppb)	ALP (K.A.units)	
	4 weeks	**8 weeks**
0	1.56 ± .82	1.48 ±0.47
50	6.11± 1.86	2.92 ± 0.91
100	6.34 ±1.21	5.45 ± 1.83
150	9.42 ± 1.01	6.59 ± 0.787
500	8.58 ± .87	6.64 ± 1.54
1000	8.3 ± 2.47	7.66 ± 1.02
2000	11.25 ±1.75	12.27± .95

Fig. 4.9. Alkaline phosphatase activity at 4 and 8 weeks of AFB_1 treatment

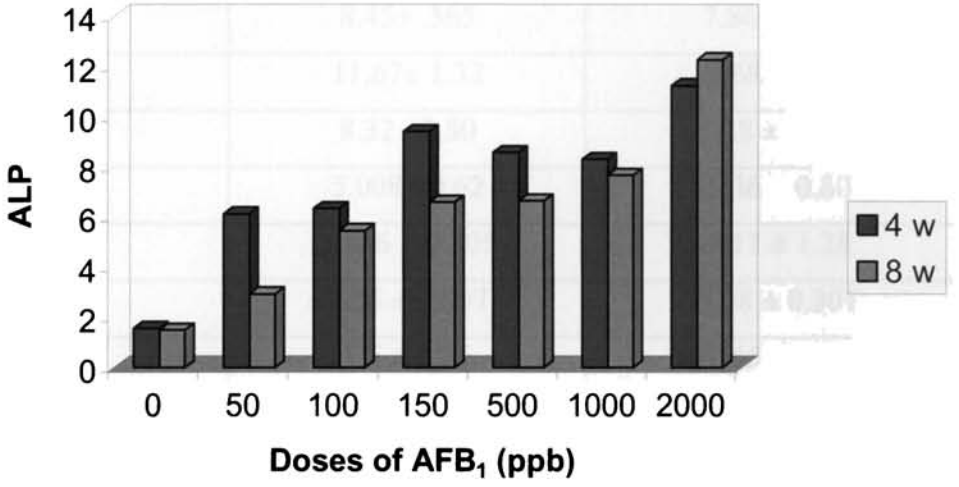


Table 4.35. ANOVA of Alkaline phosphate activity in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Dose	372.062	6	62.010	33.396	0.000
Week	15.689	1	15.689	8.450	0.007
Dose * Week	20.596	6	3.433	1.849	0.126
Error	51.990	28	1.857		

NS- Not significant

Total Serum Protein

Total serum protein (Table 4.36) levels showed high significance ($P<0.01$) between treatments but the interaction of doses at different time periods were not significant (Table 4.37). Protein and dose were negatively correlated at 4 and 8 weeks ($r = -0.877^{**}$ and -0.815^{**}).

Table 4.36. Total serum protein levels in *P. monodon* sub-adults at 4 and 8 weeks of AFB_1 exposure

Doses of AFB_1 (ppb)	Total Serum Protein	
	4 weeks	8 weeks
0	8.45± .565	7.86 ± 0 .40
50	11.67± 1.32	12.68 ± 0.453
100	8.32 ±2.80	6.18 ± 0.270
150	5.008 ±0.62	5.36 ± 0.80
500	3.866 ± 0.305	5.17 ± 1.28
1000	2.35 ± 0.507	3.78 ± 0.301
2000	1.5 ± 0.25	1.65 ± 0.83

Table 4.37. ANOVA of total serum protein in *P. monodon* at 4 and 8 weeks of AFB_1 insult

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Dose	452.990	6	75.498	73.761	0.000
Week	.566	1	.566	.553	0.463
Dose * Week	14.445	6	2.407	2.352	0.058
Error	28.660	28	1.024		
Total	2001.528	42			

Glucose

The individual effect of different doses of AFB_1 on glucose levels (Table 4.38) was highly significant ($P<0.01$). The combined effect of dose and week on glucose levels was not significant (Table 4.39). Glucose was negatively correlated with dose at 4 weeks ($r = -0.443^*$) and positive correlation was obtained ($r = 0.793^{**}$) at 8 weeks. Mean levels of glucose in the control were 18.84 mg/100 ml while the levels in the high toxin group was 30.31mg/100 ml.

Table 4.38. Glucose levels in *P. monodon* at 4 and 8 weeks of AFB_1 exposure

Doses of AFB_1 (ppb)	Glucose	
	4 weeks	**8 weeks**
0	18.84± 1.14	20.61± 1.67
50	22.86± 4.64	22.56±2.64
100	15.36± 6.97	23.4± 4.0
150	29.31± 3.49	25.39± 4.22
500	22.88± 3.99	26.03 ±1.56
1000	28.11± 1.91	27.62± 1.822
2000	27.95 ±3.8	30.31± 1.90

Table 4.39. ANOVA of glucose levels in *P. monodon* fed AFB_1 diets at 4 and 8 weeks

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Dose	550.158	6	91.693	7.599	0.000
Week	20.259	1	20.259	1.679	0.206
Dose * Week	129.935	6	21.656	1.795	0.136
Error	337.840	28	12.066		
Total	26127.807	42			

NS- Not Significant

Cholesterol

The cholesterol levels (Table 4.40) were highly significant ($P<0.01$) between dose and week (Fig. 4.10). Cholesterol levels in 2000 ppb group were significantly ($P<0.01$) higher than other groups (Table 4.41). DMRT revealed high level of significance between 50 ppb and 2000 groups. Negative correlation was noticed after 4 and 8 weeks between AFB_1 doses and cholesterol levels (r= -0.299, -0.443*).

Table.4.40. Serum cholesterol levels in *P. monodon* sub-adults fed AFB_1 contaminated diets at 4 and 8 weeks

Doses of AFB_1 (ppb)	Cholestrol	
	4 weeks	8 weeks
0	55.64± 3.32	37.83 ±2.75
50	80.24 ±2.0	84.34± 3.67
100	57.83± 1.74	48.28± 1.86
150	62.03± 2.98	54.27± 3.98
500	54.69 ±5.61	46.64± 2.84
1000	45.48 ± 4.25	51.23± 3.5
2000	65.29 ±3.04	25.35 ±3.95

Fig. 4.10. Serum cholesterol levels in *P. monodon* sub-adults fed AFB_1 diets at 4 and 8 weeks

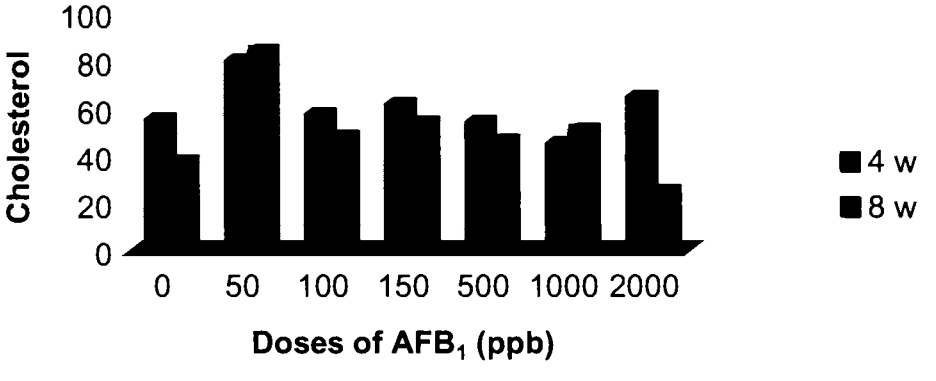


Table 4.41. ANOVA of cholesterol levels in *P. monodon* fed AFB_1 diets at 4 and 8 weeks

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	126786.137	1	126786.137	1765.481	.000
Dose	5900.294	6	983.382	13.693	.000
Week	1150.077	1	1150.077	16.015	.000

Significant at ($P<0.01$)

Aflatoxin B_1 residue in shrimp carcass

The aflatoxin B_1 residue levels present in the shrimp carcass in the different treatment groups are presented in the table 4.42. No aflatoxin residues were present in the control, 50 ppb group, 100 ppb and 150 ppb toxin dosed group. AFB_1 doses showed positive correlation with the toxic residue in the shrimp body.

Table 4.42. Aflatoxin B_1 residues in the carcass of different groups of shrimps at 8 weeks

AFB_1 Doses (ppb)	AFB_1 residue (ppb)
0	0
50	0
100	0
150	0
500	74
1000	83.3
2000	129.16

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Histology

The cephalothorax region of the shrimps from control and AFB_1 treatment groups was taken for histological study. The cephalothorax of the control shrimp revealed normal architecture. Hepatopancreas alone showed severe changes in the treatment groups. The lymphoid organ, mandibular organ and antennal gland revealed only mild lysis. Hepatopancreas was selected for detailed examination since it was the target organ most affected.

In the 50 ppb treatment group at 4 weeks there was lysis of tubules and loss of architecture in few areas, more lysis was noticed in the apical and middle region of the hepatopancreas (Plate11a-11c). No significant change was observed in lymphoid organ and the antennal gland. After 8 weeks necrosis, fibrosis and cell destruction were observed in some regions

Plate 11

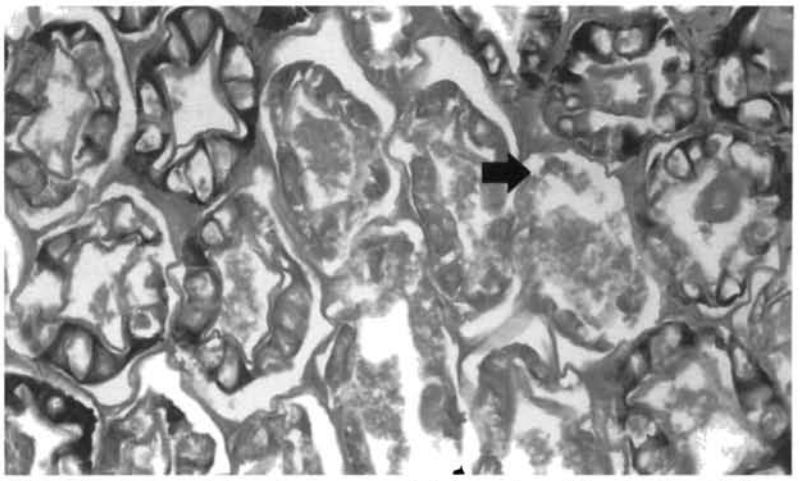


Plate 11a. Section of hepatopancreas of shrimp given 50 ppb aflatoxin B_1 in the diet at 4 weeks revealing change in structure of tubules (arrow) and loss of brush border appearance (arrow head). H&E 200x

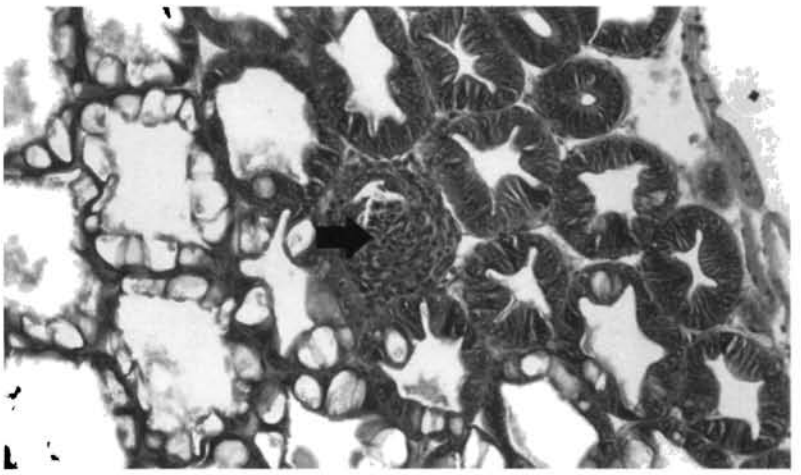


Plate 11b. Hepatopancreas of 50 ppb aflatoxin B_1 fed shrimp at 4 weeks. Note the haemocytic nodule formation in the tubules (arrow). H&E 200x

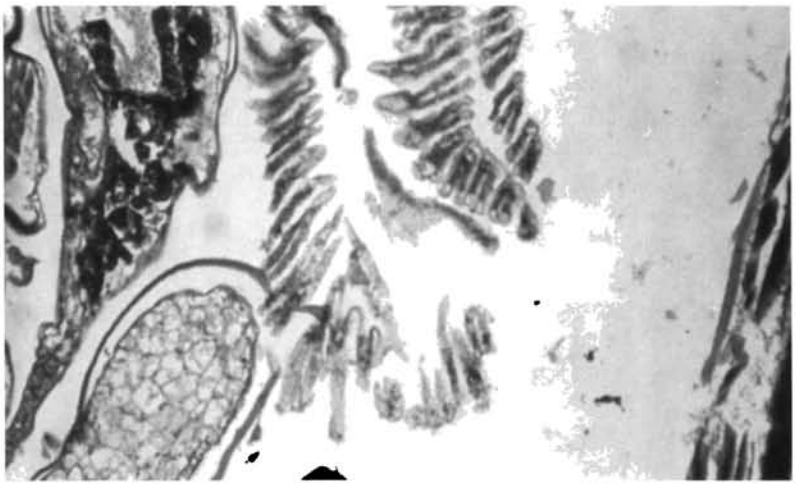


Plate 11c. Section of shrimp revealing mild necrosis in the gill region and muscles in shrimp given 50 ppb aflatoxin B_1 at 4 weeks. H&E 200x

of the hepatopancreas. No change was observed in the lymphoid organ. Mild necrosis was noticed in the antennal gland (Plate 12a-12c).

The hepatopancreas of shrimps in the 100 ppb AFB_1 group after 4 weeks revealed more fibrosis around the tubules, melanised nodules and necrotic cells in the lumen (Plate 13a). There was desquamation and thickening of intertubular tissue. Formation of peculiar elongated cells and destruction of E cells were observed. R cells have almost disappeared in some areas (Plate 13b-13c). After 8 weeks, severe necrosis and fibrosis were found. There was reduction in the number of R cells and B cells in the hepatopancreas (Plate 14 a-14c).

In the shrimps treated with 150 ppb AFB_1 after 4 weeks there was complete loss of structure of cells and tubules of the hepatopancreas. Necrotic cells were seen in the lumen and loss of brush bordered appearance noticed. Complete detachment of cells was observed. There was degeneration of focal areas and beginning of fibrous tissue growth (Plate 15a-15b). After 8 weeks, necrosis and lysis became extensive; more fibrous growth and desquamated cells were noticed in the lumen; and inflammatory reaction was observed in between lobules (Plate 15c).

Shrimps fed with 500 ppb AFB_1 after 4 weeks revealed extensive fibrosis, degeneration, cell elongation and loss of cells in the distal end of the hepatopancreas (Plate 16a-16b). After 8 weeks, the hepatopancreas showed destruction of the tubular structure in the distal region and necrosis (Plate 16c).

In the shrimps dosed with 1000 ppb AFB_1, inflammatory reaction and cell elongation were the peculiar features observed in the hepatopancreas. Fibrosis, necrosis and degeneration were intense (Plate17a-17c). After 8 weeks, many lumen interconnections, cystic hyperplasia and dilation were noticed. There was necrosis of antennal gland and inflammatory reaction in lymphoid organ. Other changes observed in the hepatopancreas were fibrous

Plate 12

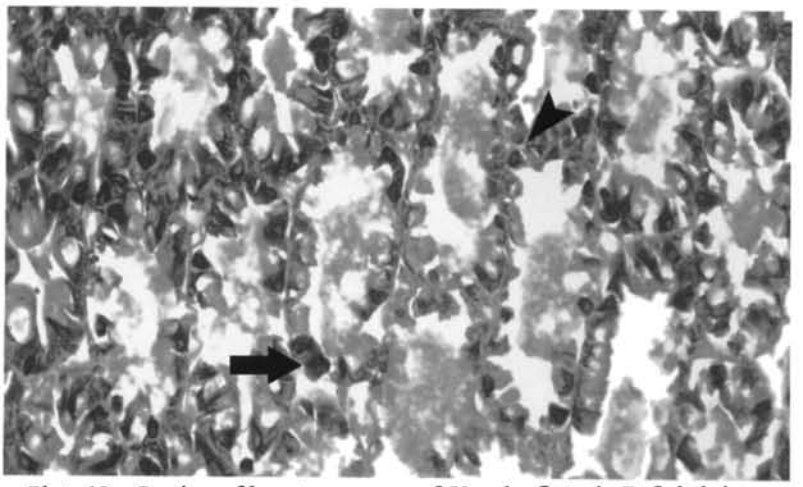


Plate 12a. Section of hepatopancreas of 50 ppb aflatoxin B_1 fed shrimp after 8 weeks showing desquamation of tubules (arrow) and loss of cells (arrow head). H&E 200x

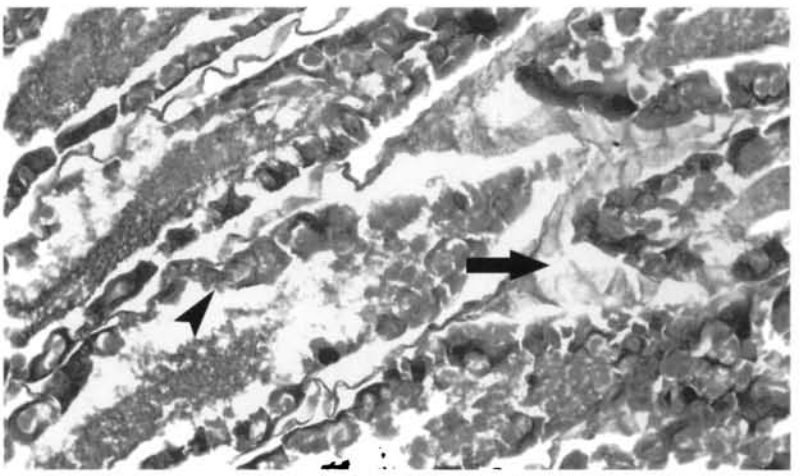


Plate 12b. Hepatopancreas section of 50 ppb aflatoxin B_1 dosed shrimp after 8 weeks revealing necrosis (arrow) and desquamation (arrow head). H&E 400x

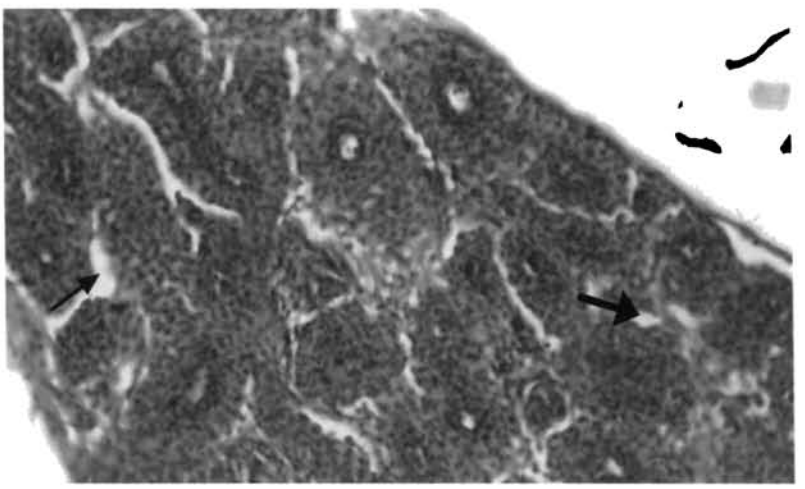


Plate 12c. Section revealing mild vacuolation in the lymphoid organ of shrimp given 50 ppb aflatoxin B_1 at 4 weeks. H&E 200x

Plate 13

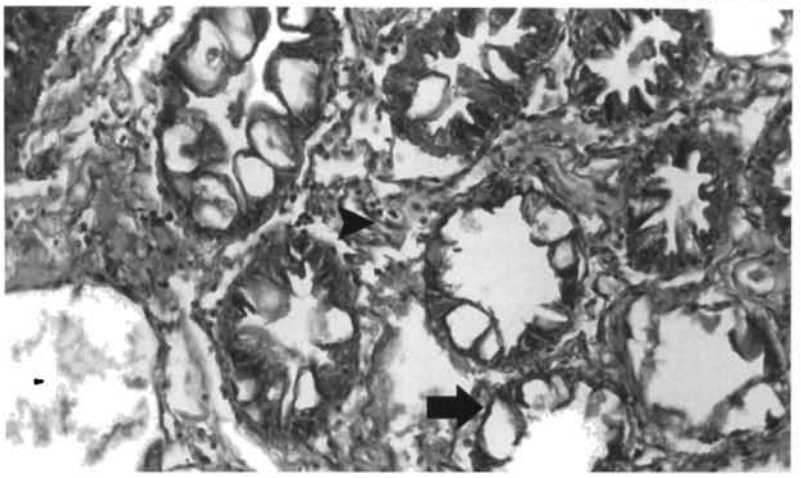


Plate 13a. Hepatopancreas of shrimp given 100 ppb aflatoxin B_1 at 4 weeks. Note the cellular inflammatory response (arrow head) and loss of cells (arrow). H&E 200x

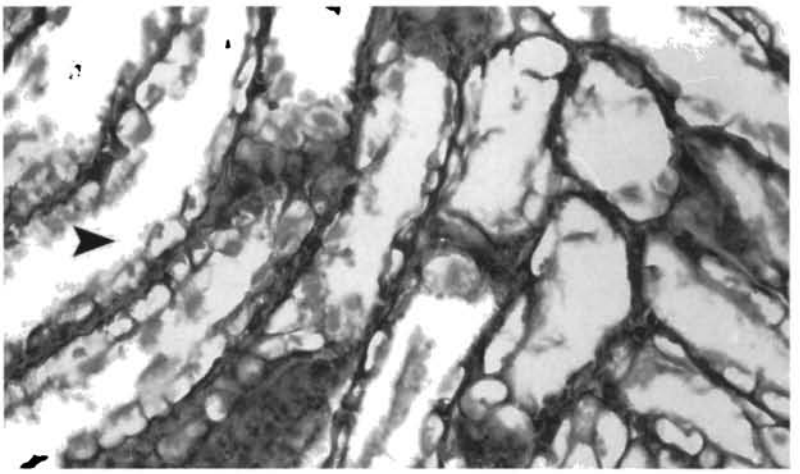


Plate13b. Section of hepatopancreas of shrimp fed 100 ppb aflatoxin B_1 at 4 weeks revealing elongation of the tubules and loss of cells(arrow head). H&E 200x

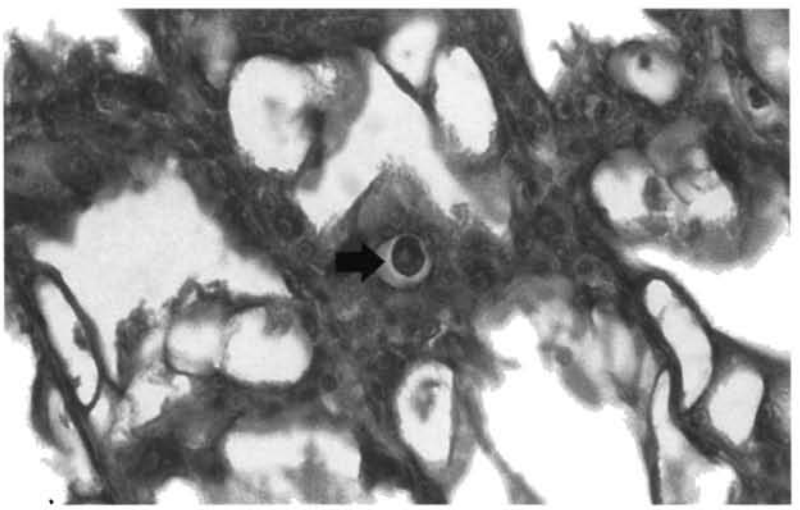


Plate 13c. Haemocytic infiltration (arrow) revealed in the hepatopancreas section of shrimp given 100 ppb aflatoxin B_1 at 4 weeks. H&E 400x

Plate 14

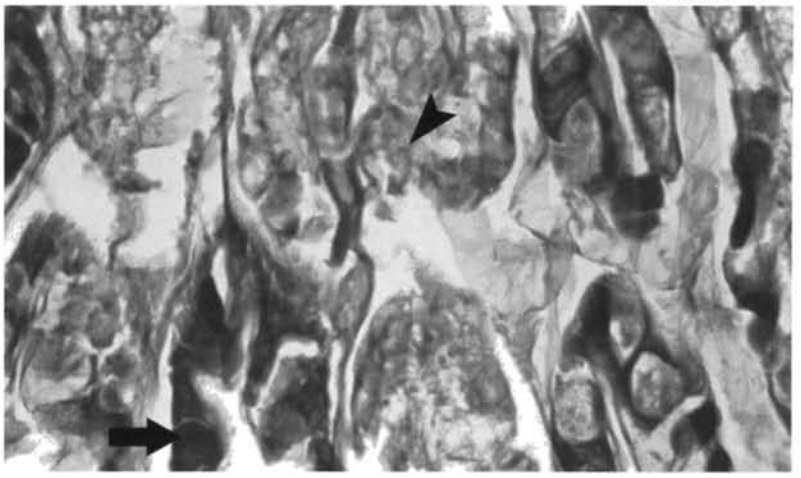


Plate 14a. Section of hepatopancreas of 100 ppb aflatoxin B_1 treated shrimp at 8 weeks revealing rounding of cells (arrow) and loss of cells (arrow head). H&E 200x

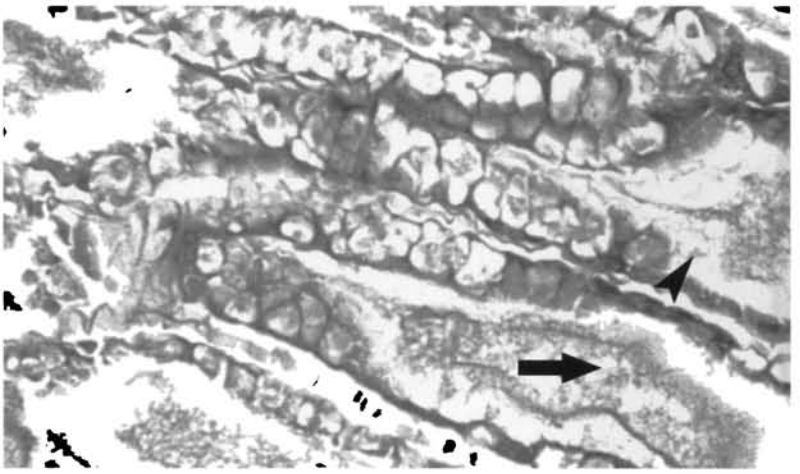


Plate 14b. Section of heaptopancreas of 100 ppb aflatoxin B_1 treated group at 8 weeks. Note the necrotic changes (arrow) and loss of tubules (arrow head). H&E 200x

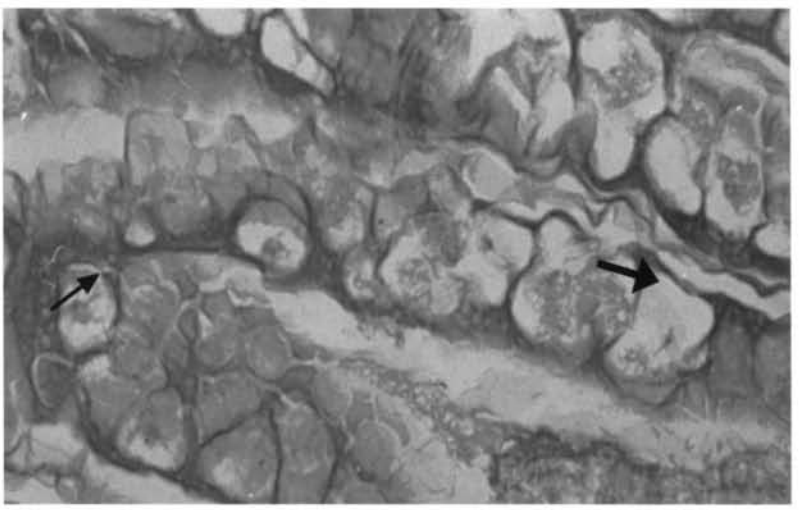


Plate 14c. Enlarged view of the hepatopancreas of shrimp given 100 ppb aflatoxin B_1 diet showing necrosis. H&E 400x

Plate 15

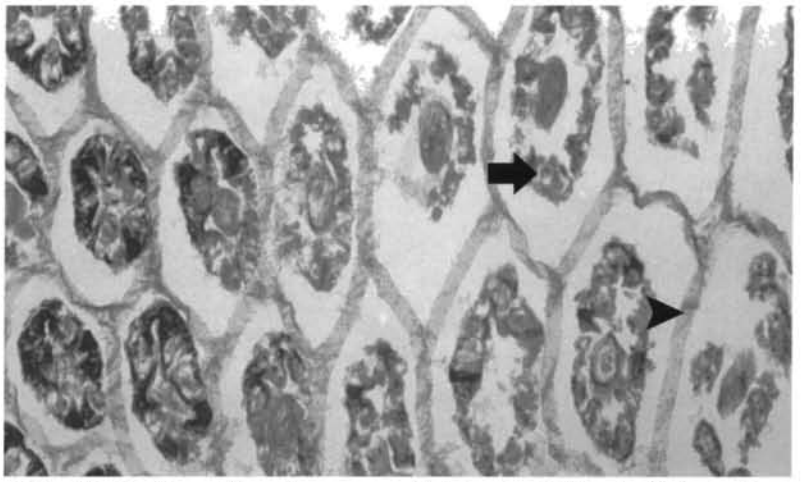


Plate 15a. Section of hepatopancreas of shrimp fed 150 ppb aflatoxin B_1 at 4 weeks showing fibrous growth (arrow head) and desquamated cells (arrow). H&E 200x

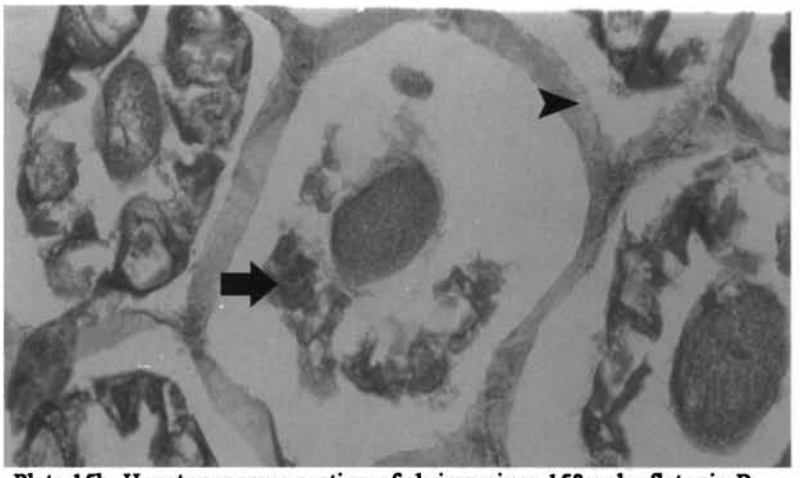


Plate 15b. Hepatopancreas section of shrimp given 150 ppb aflatoxin B_1 at 4 weeks revealing an enlarged view of completely desquamated cell (arrow) and fibrosis (arrow head). H&E 400x

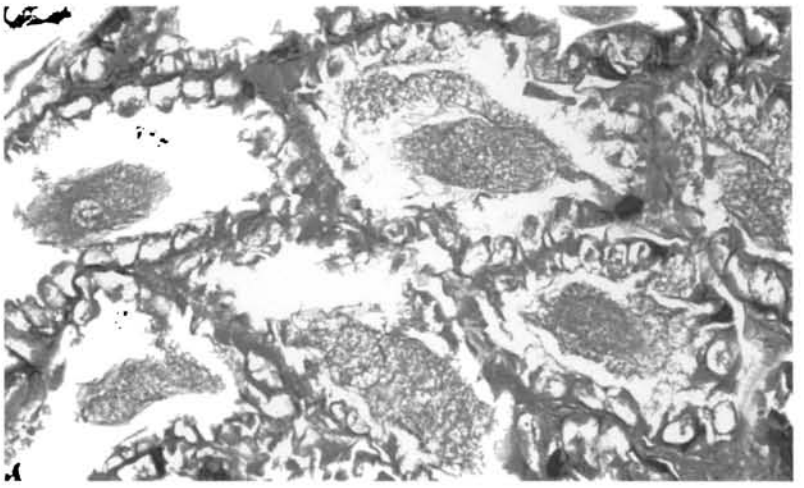


Plate 15c. Section of hepatopancreas of 150 ppb aflatoxin B_1 treated shrimp at 8 weeks. Note the replacement of cells by fibrous growth. H&E 200x

Plate 16

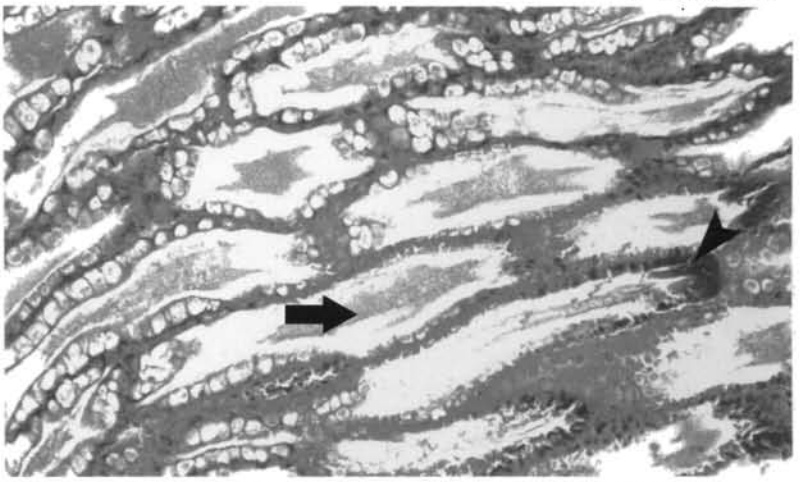


Plate 16a. Hepatopancreas of 500 ppb AFB_1 given shrimp at 4 weeks showing extensive fibrosis (arrow head) and cell elongation (arrow). H&E 100x

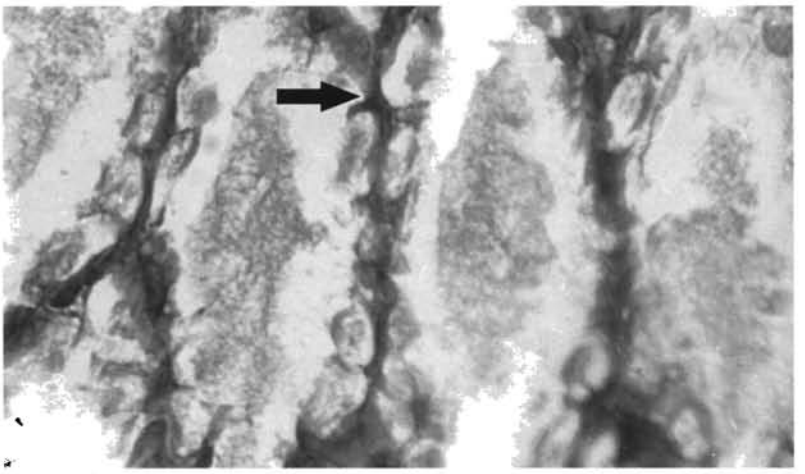


Plate 16b. Enlarged view of section of hepatopancreas of shrimp fed 500 ppb AFB_1 at 4 weeks. Note a degenerative tubule without cells. H&E 400x

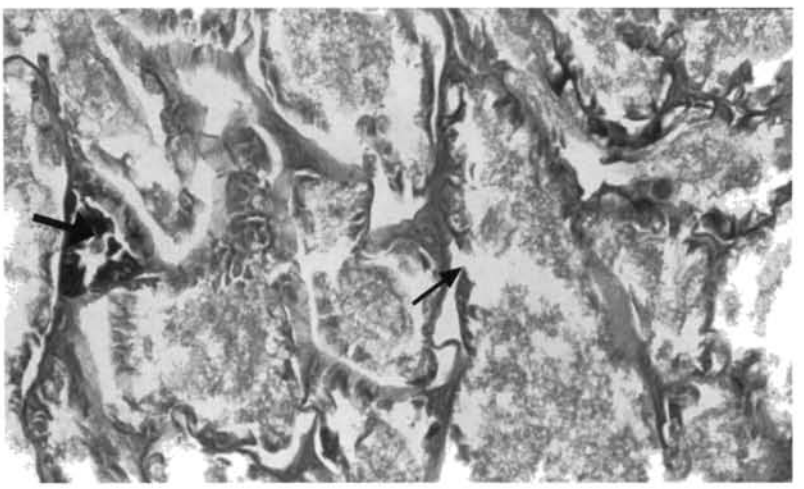


Plate 16c. Section revealing a completely necrosed area in the hepatopancreas of 500 ppb aflatoxin B_1 treated shrimp at 8 weeks. H&E 200x

Plate 17

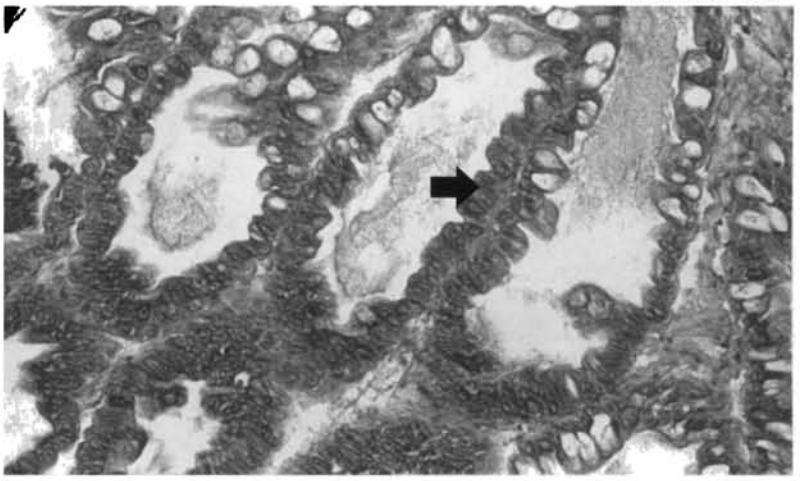


Plate 17a. Hepatopancreas of shrimp fed 1000 ppb aflatoxin B_1 at 4 weeks revealing severe fibrosis of the tubules. H&E 200x

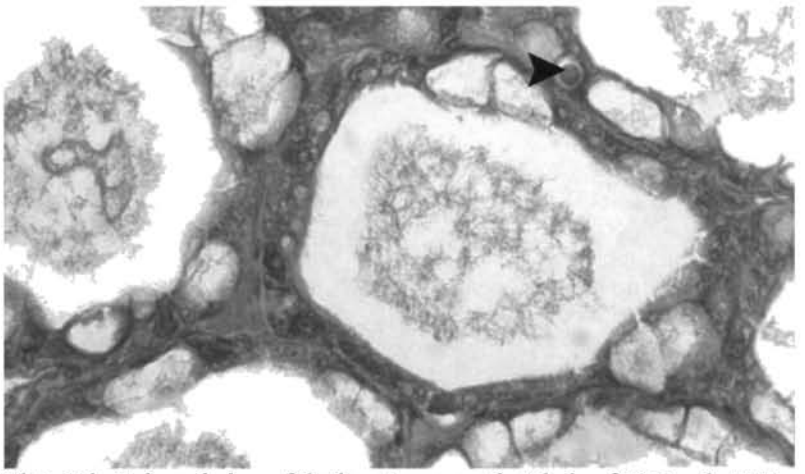


Plate 17b. Enlarged view of the hepatopancreatic tubule of 1000 ppb AFB_1 treated shrimp showing haemocytic infiltration (arrow head). H&E 400x

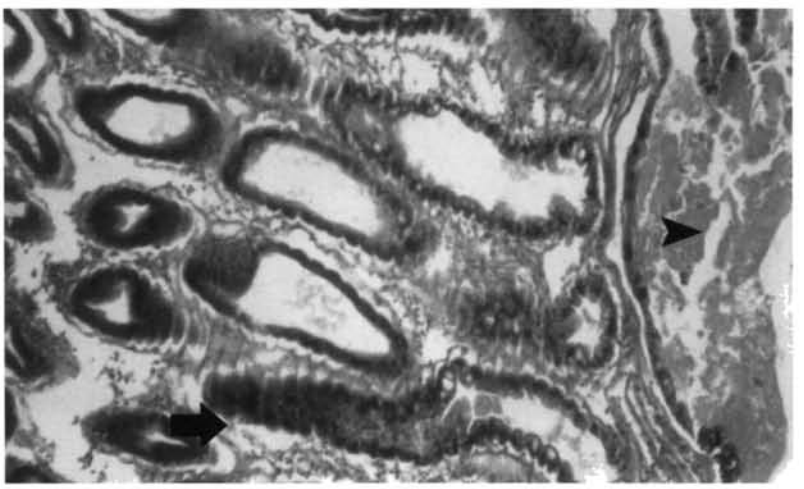


Plate 17c. Necrosis (arrow head) and fibrosis (arrow) observed in the section of hepatopancreas of 1000 ppb AFB_1 fed shrimp at 4 weeks. H&E 200x

Plate 18

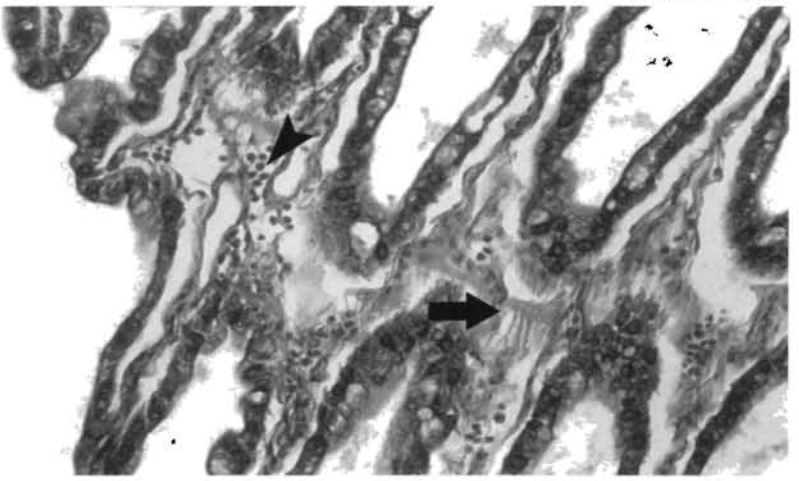


Plate 18a. Hepatopancreas of 1000 ppb AFB_1 treated shrimp at 8 weeks revealing inflammatory response (arrow head) and tubule elongation (arrow). H&E 200x

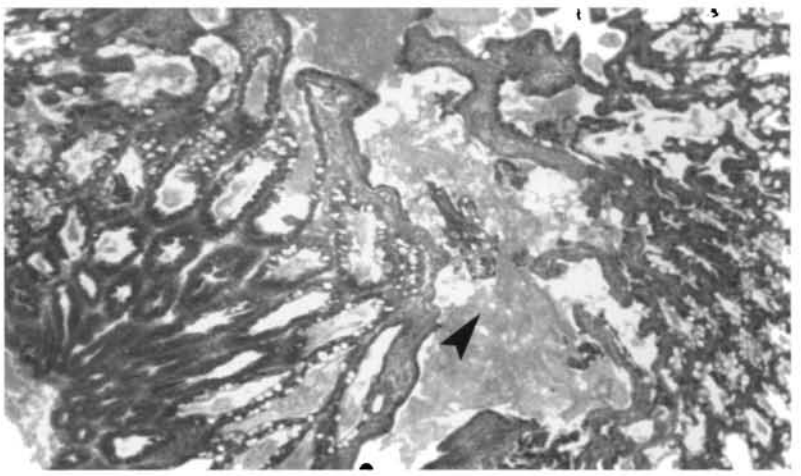


Plate 18b. Severe necrosis in the section of the hepatopancreas of 1000 ppb aflatoxin B_1 dosed shrimp at 8 weeks. H&E 100x

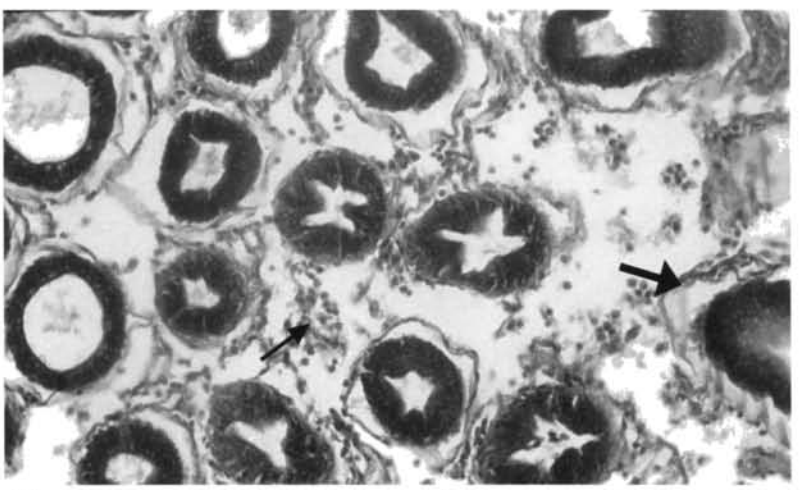


Plate 18c. Section of the hepatopancreas showing inflammatory response and cell detachment in shrimp fed 1000 ppb aflatoxin B_1 at 8 weeks. H&E 200x

Plate 19

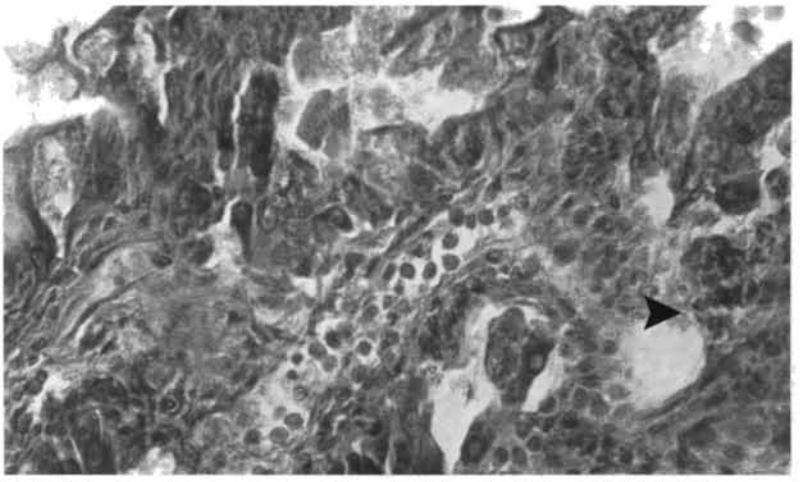


Plate 19a. Hepatopancreas section of 2000 ppb aflatoxin B_1 fed shrimp showing severe necrosis at 4 weeks. H&E 400x

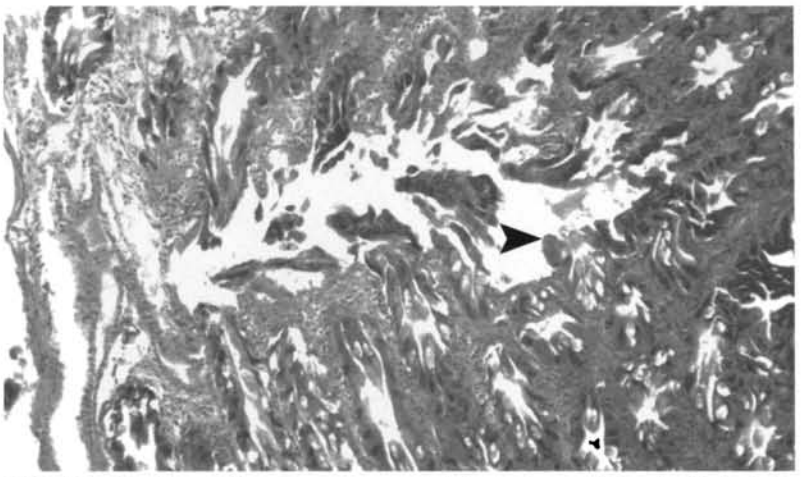


Plate 19b. Section of hepatopancreas of shrimp given 2000 ppb aflatoxin B_1 showing atrophy of the tubules. H&E 100x

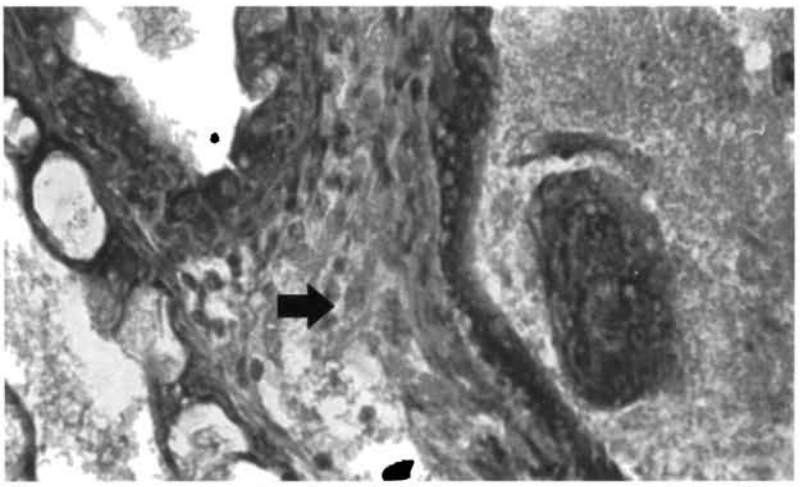


Plate 19c. Severe necrosis and cellular inflammatory response observed in the section of hepatopancreas of the shrimp given 2000 ppb AFB_1. H&E 400x

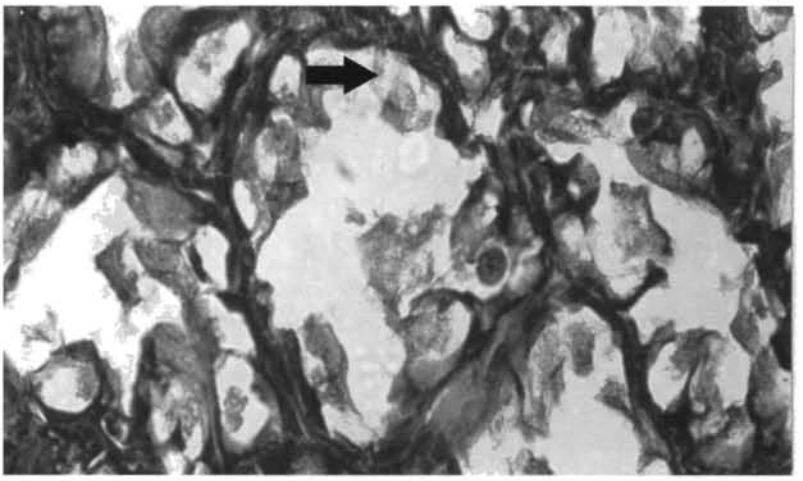


Plate 20a. Note the loss of cells in the tubules of hepatopancreas (arrow), haemocytic infiltration (arrow head) in shrimp fed 2000 ppb aflatoxin B_1 at 8 weeks. H&E 400x

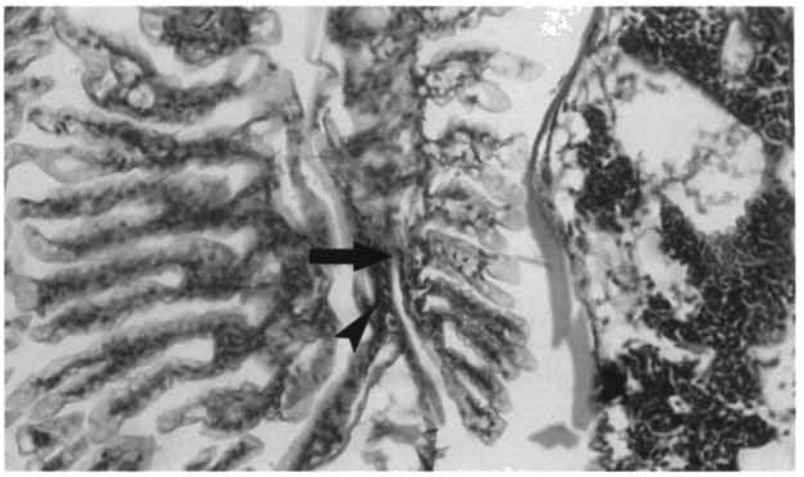


Plate 20b. Section of the gills revealing necrosis at 8 weeks in shrimp fed with 2000 ppb aflatoxin B_1. H&E 400x

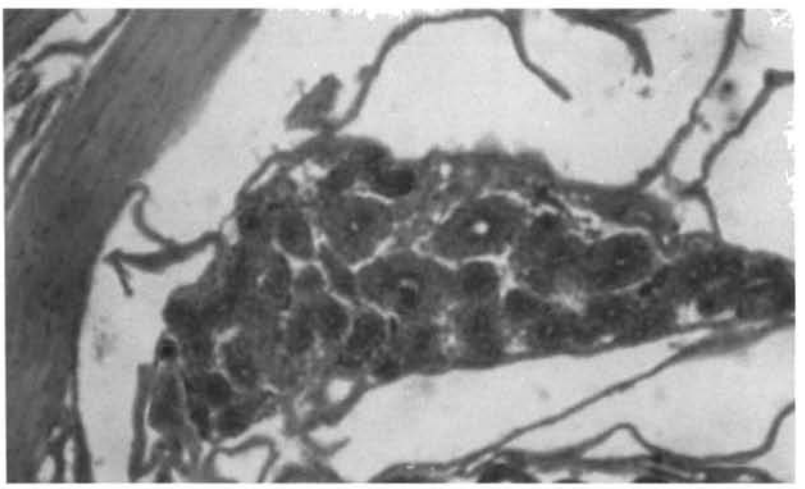


Plate 20c. Section showing vacuolation in the lymphoid organ in shrimp fed 2000 ppb AFB_1. H&E 100x

tissue growth around the tubules, haemocytic infiltration and proliferation of cells (Plate 18a-18c).

Shrimps fed with 2000 ppb AFB_1 histologically showed severe necrosis, extensive fibrosis, fibrous tissue growth, haemocytic infiltration and intense papillomatous growth in the hepatopancreas (Plate 19a-19c). After 8 weeks, cellular inflammatory response was observed. There was complete loss of architecture of the entire focal area. Fibrous tissue growth has replaced the tubules and cells. Apoptosis or rounding of cells was also observed in few areas (Plate 20 a-20c).

Ultrastructure

Hepatopancreas of shrimps from the control group, 1000 ppb and 2000 ppb AFB_1 treatments after 4 and 8 weeks, were taken for ultrastructural examination. The electronmicroscopic view of the control group showed normal architecture of the cells with nucleus, well-developed smooth and rough endoplasmic reticulum. In between the rough endoplasmic reticulum (RER) and the smooth endoplasmic reticulum (SER), round and rod shaped mitochondria with numerous cristae and granules were seen. The nucleus appeared spherical with abundant euchromatin and well developed nucleolus. The golgi bodies and microvilli were normal (Plate 21a-21d).

Hepatopancreas of the shrimp exposed to 1000 ppb AFB_1 group for 4 weeks revealed fragmentation of endoplasmic reticulum with small dilatations and accumulation of densities. Changes were noticed in the nucleus with the condensation of chromatin to nuclear membrane. There was vacoulation in the cytoplasm. The nucleus contained chromatin granules and electron dense inclusions. The cell membrane was broken abruptly. Shapes of mitochondria were affected with change in structure of the cristae. Microvilli were broken at few places. Extensive vacuolation was

Plate 21

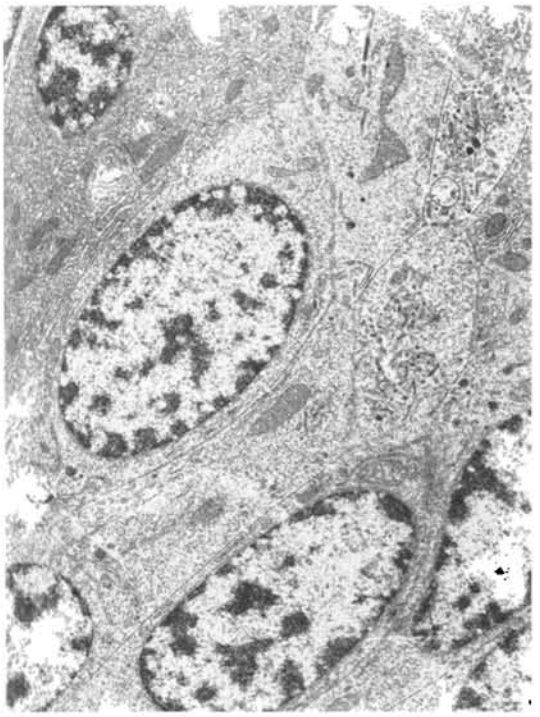


Plate 21a. Electron micrograph depicting the gross view of cells in the hepatopancreas of control *P. monodon*. 5000x

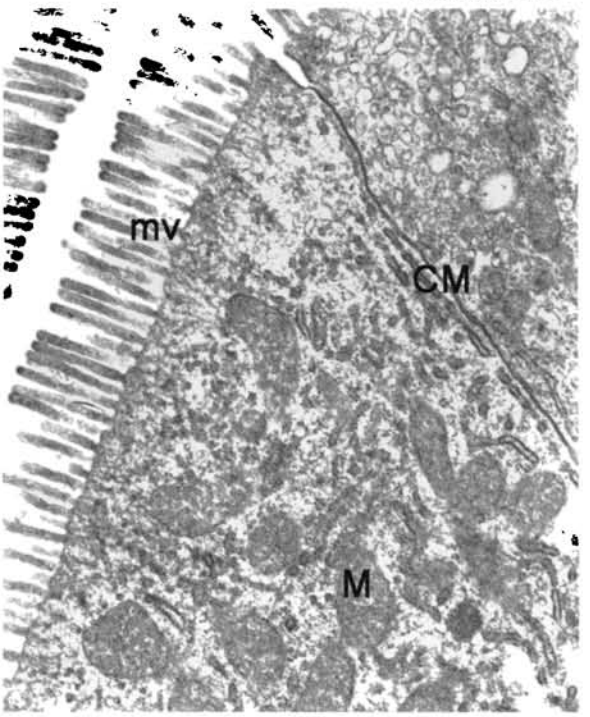


Plate 21b. The ultrastructural view of hepatopancreas of control shrimp showing intact cell membrane, large number of mitochondria (M) and microvilli (mv) projecting into the lumen. 15000x

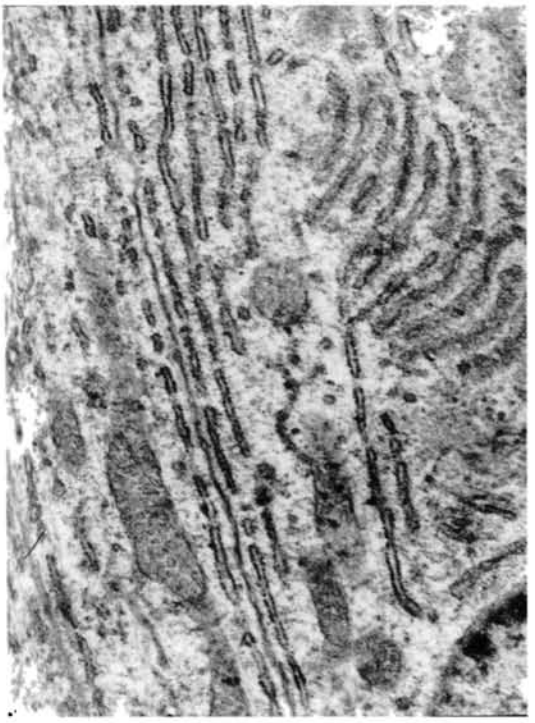


Plate 21c. Electron micrograph revealing rough endoplasmic reticulum, smooth endoplasmic reticulum and mitochondria in the hepatopancreas of shrimp of control group. 10000x

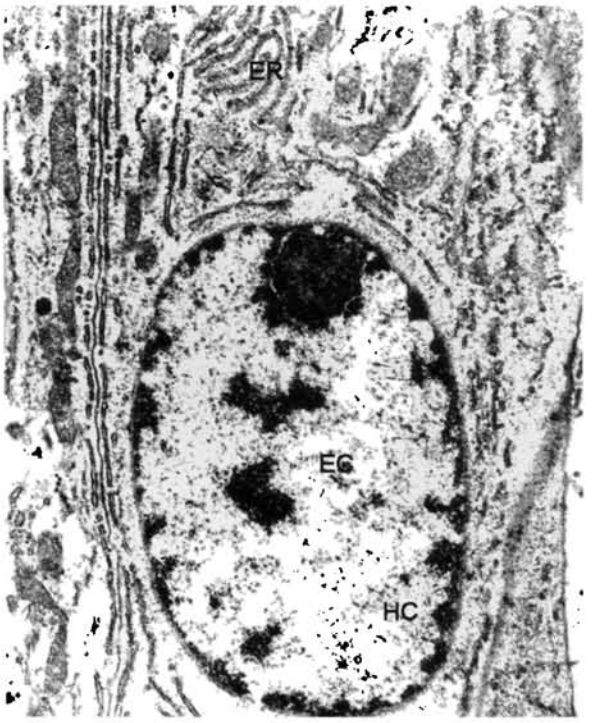


Plate 21d. Electron micrograph of the heptopancreas of control *P. monodon* depicting nuclues with euchromatin (EC) and heterochromatin (HC)attached to nuclear membrane. Note ER cisternae and cell membrane. 10000x

Plate 22

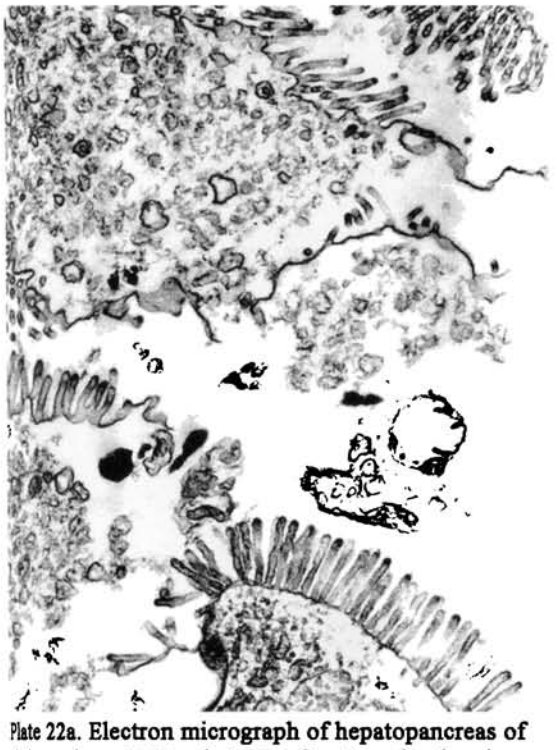


Plate 22a. Electron micrograph of hepatopancreas of shrimp given 1000 ppb AFB_1 after 4 weeks showing disintegration of microvilli (arrow). 10000x

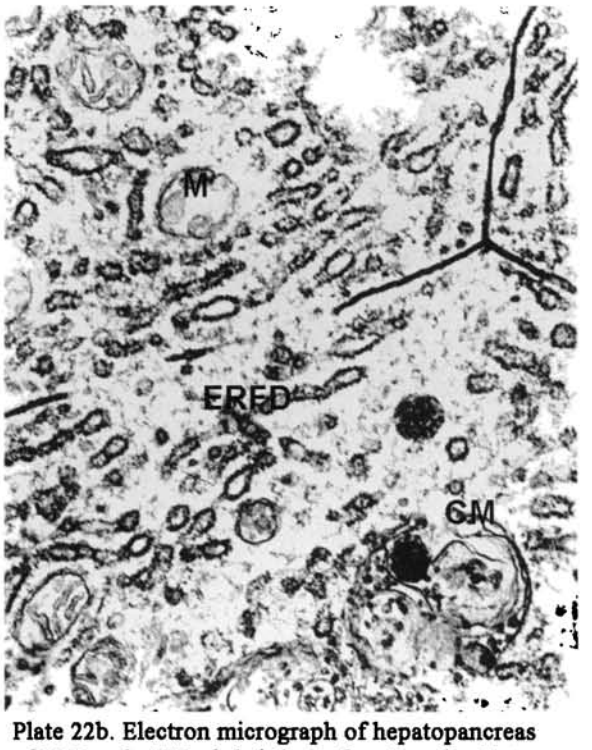


Plate 22b. Electron micrograph of hepatopancreas of 1000 ppb AFB_1 fed shrimp after 4 weeks. Note the ER fragmentation with small dilations (ERFD) swollen mitochondria (SM) and condensation of mitochondria(CM). 20000 x

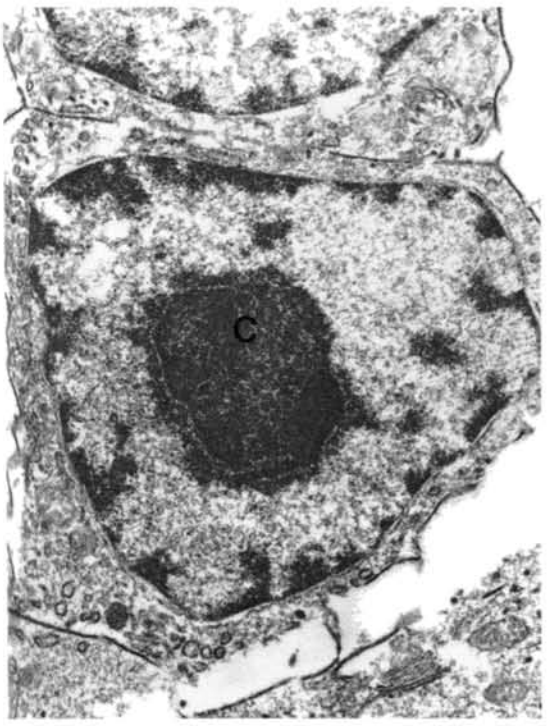


Plate 22c. Electron micrograph of hepatopancreas of 1000 ppb AFB_1 dosed shrimp at 4 weeks showing condensation of chromatin (C) in the nucleus.10000 x

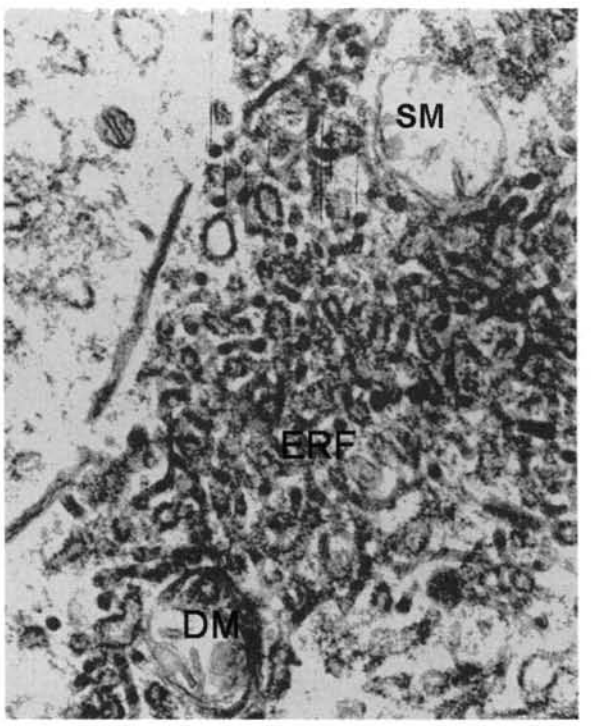
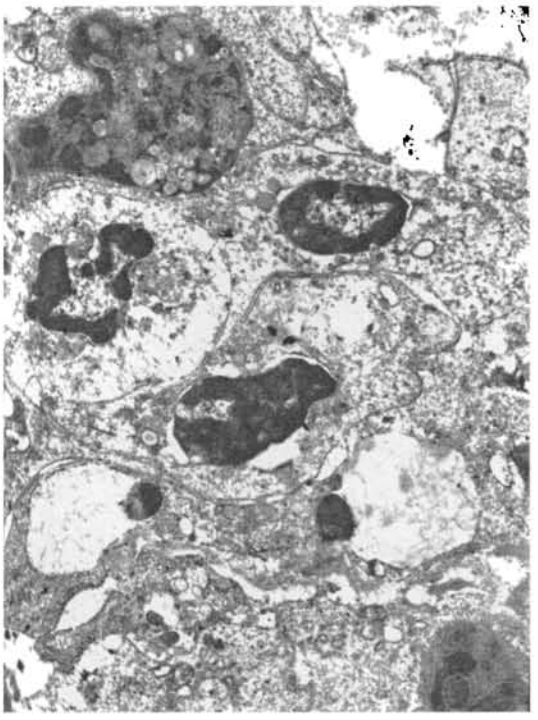


Plate 22d. Electron micrograph of hepatopancreas of shrimp given 1000 ppb AFB_1 after 4 weeks depicting fragmentation of ER (ERF) with dilation and accumulation of densities, degranulation of mitochondria and broken cell wall. 25000x



late 23a. Electron micrograph depicting extensive acuolation in the hepatopancreas of shrimp fed 000 ppb AFB_1 at 8 weeks. 15000x

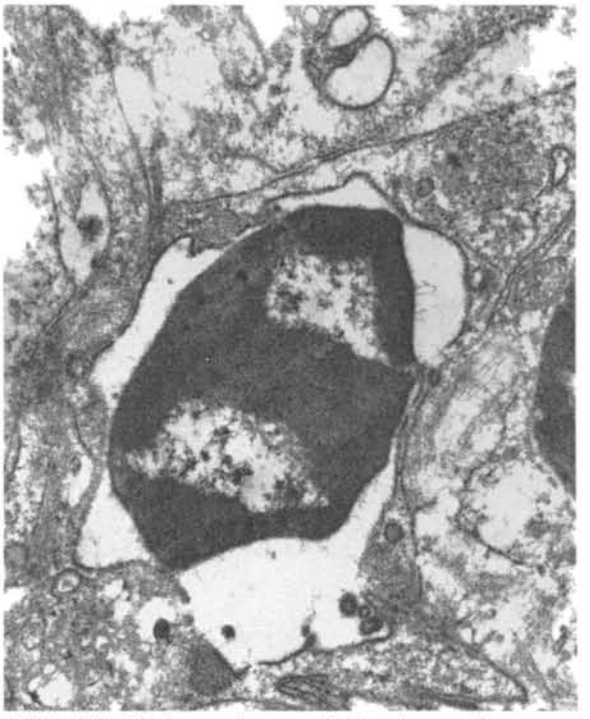
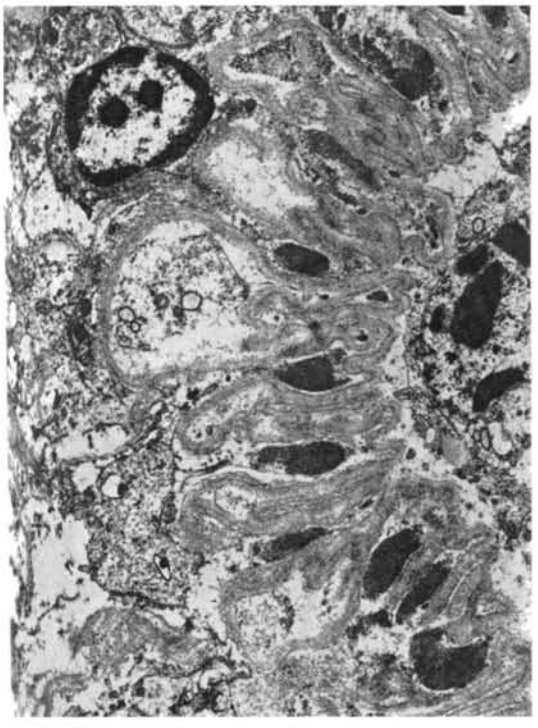


Plate 23b. Electron micrograph showing nuclear vacuolation (NV) in the hepatopancreas of shrimp given 1000 ppb AFB_1 at 8 weeks. 15000x



'ate 23c. Electron micrograph depicting the rounding f cells in the hepatopancreas of shrimp dosed 00 ppb AFB_1 at 8 weeks. 8000x

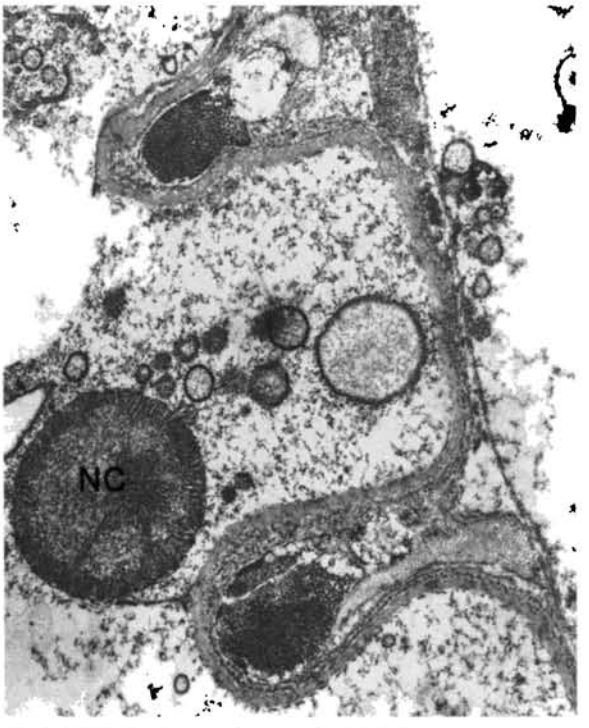


Plate 23d. Electron micrograph of shrimp hepatopancreas given 1000 ppb aflatoxin B_1 at 8 weeks revealing nuclear condensation (NC) and granulation of endoplasmic reticulum (ERG). 15000x

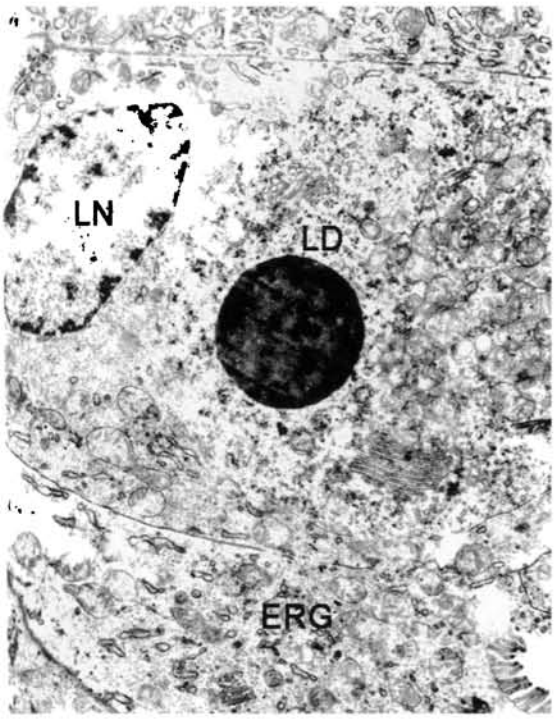


Plate 24a. Electron micrograph of hepatopancreas of shrimp fed 2000 ppb AFB_1 group at 4 weeks showing lipid droplet(LD), granulation of endoplasmic reticulum (ERG) and loss of nuclear contents (LN). 6000x

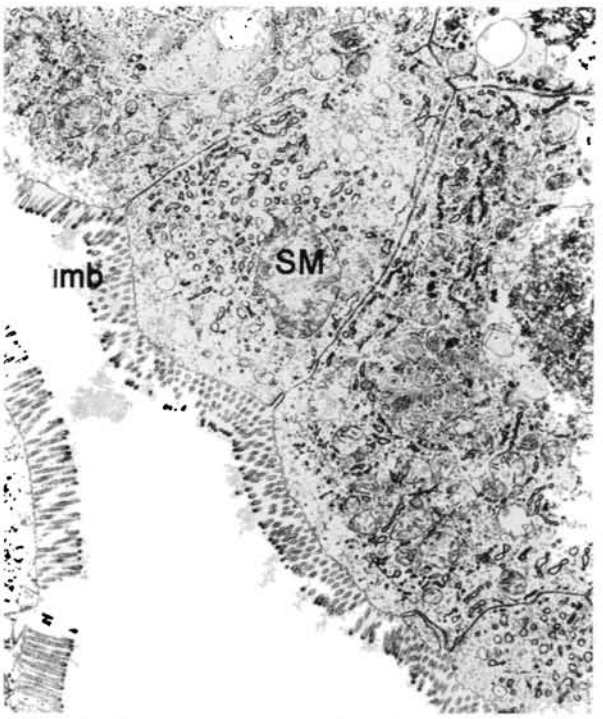


Plate 24b. Electron micrograph showing the rupture of microvillus border (lmb) into the lumen of the hepatopancreas. Note the swollen mitochondria (SM) in the section. 5000x

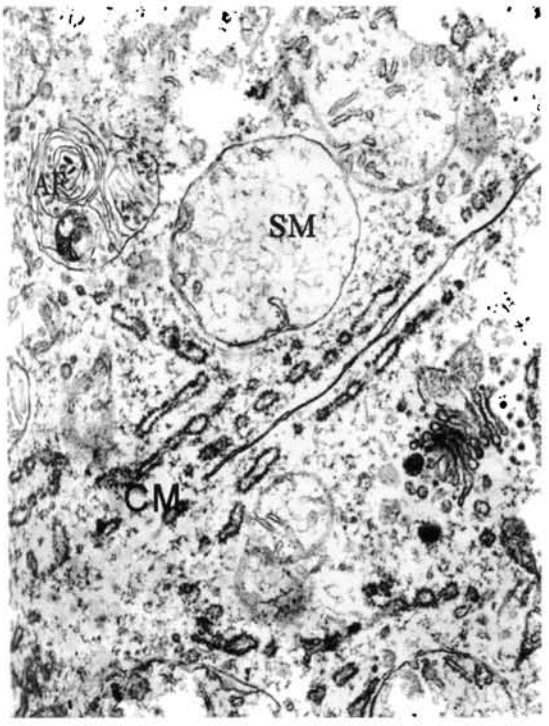


Plate 24c. Electron micrograph of the hepatopancreas of shrimp given 2000 ppb aflatoxin B_1 at 4 weeks. Note the broken cell membrane (CM), swollen mitochondria (SM) and autophagy (AF). 80000x

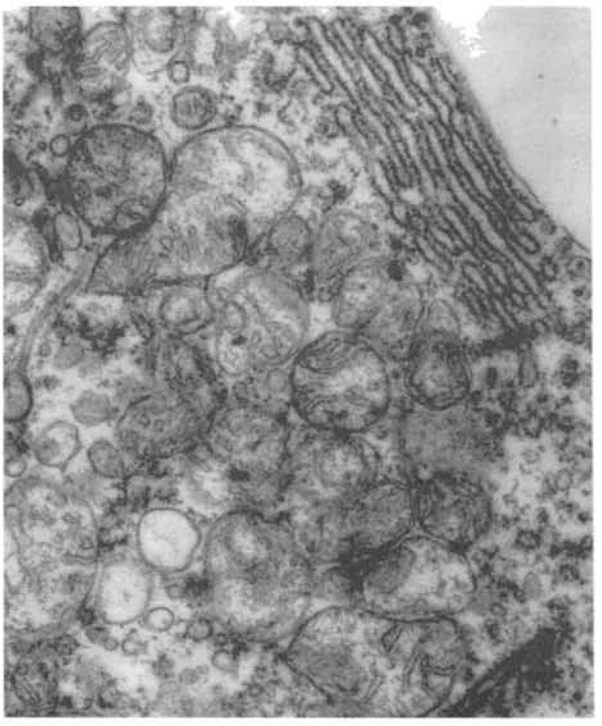


Plate 24d. Ultrastructural view of hepatopancreas of shrimp fed 2000 ppb AFB_1 at 4 weeks. Note the swollen mitochondia with loss of cristae. 20000x

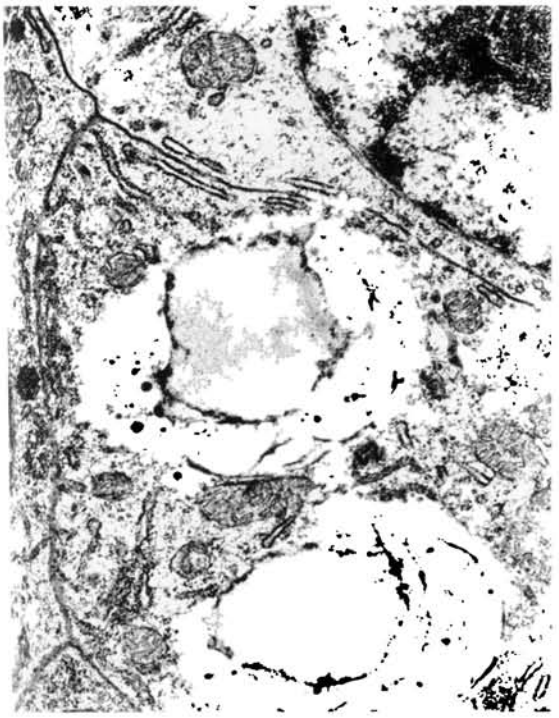


Plate 25a. Electron micrograph of hepatopancreas of shrimp treated 2000 ppb aflatoxin B_1 at 8 weeks revealing vacuole formation. 6000 x

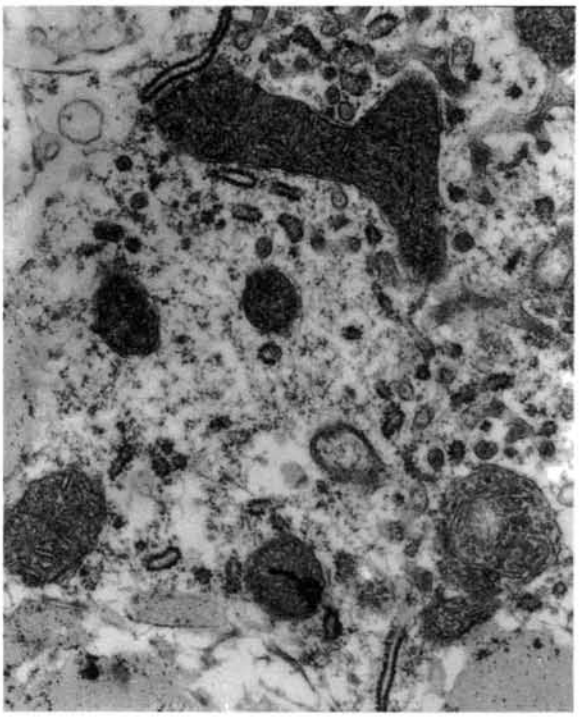


Plate 25b. Electron micrograph depicting change in the configuration of mitochondria. 10000x

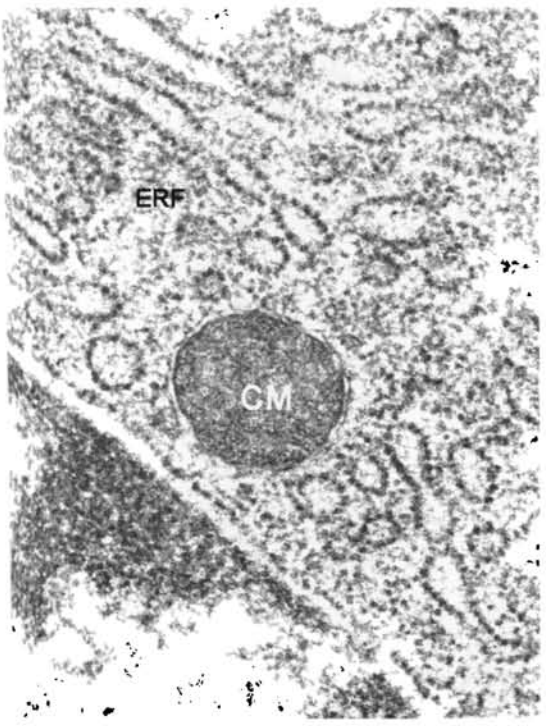


Plate 25c. Electron micrograph depicting condensation of mitochondria (CM) and fragmentation of endoplasmic reticulum (ERF). 20000x

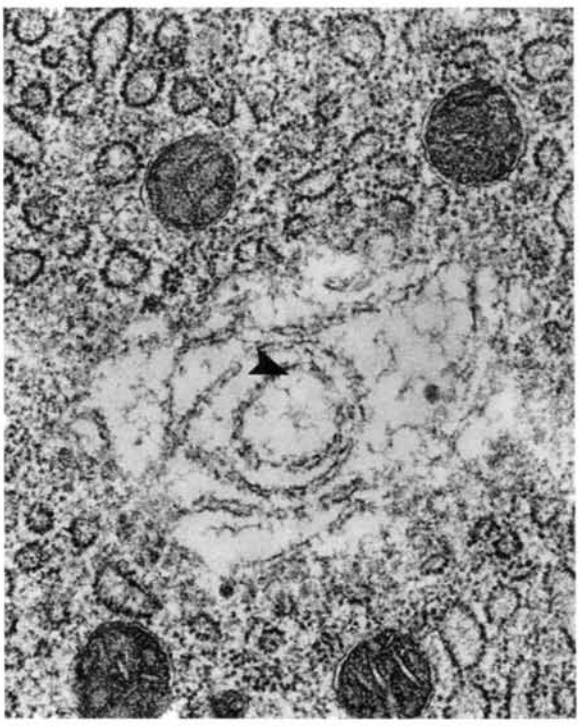


Plate 25d. Electron micrograph revealing whorl formation in the hepatopancreas of shrimp fed 2000 ppb AFB_1 at 8 weeks. 15000 x

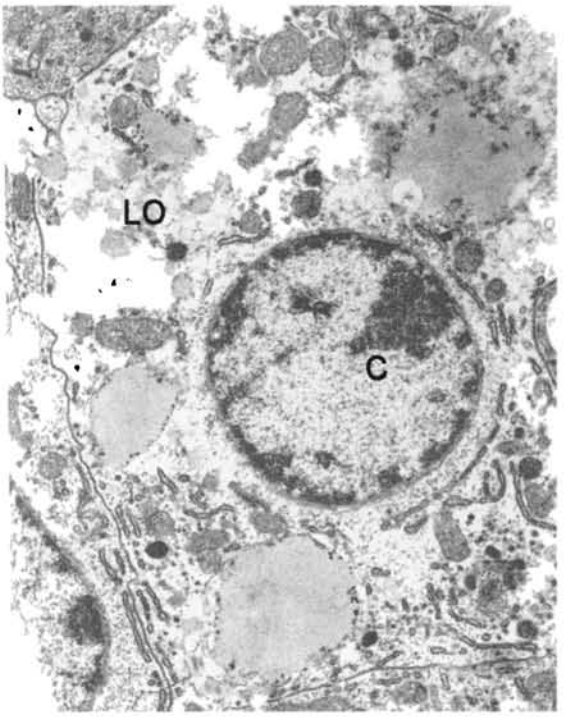


Plate 26a. Electron micrograph depicting the chromatin condensation in the nucleus (C) and loss of cell organelles (LO). 3000x

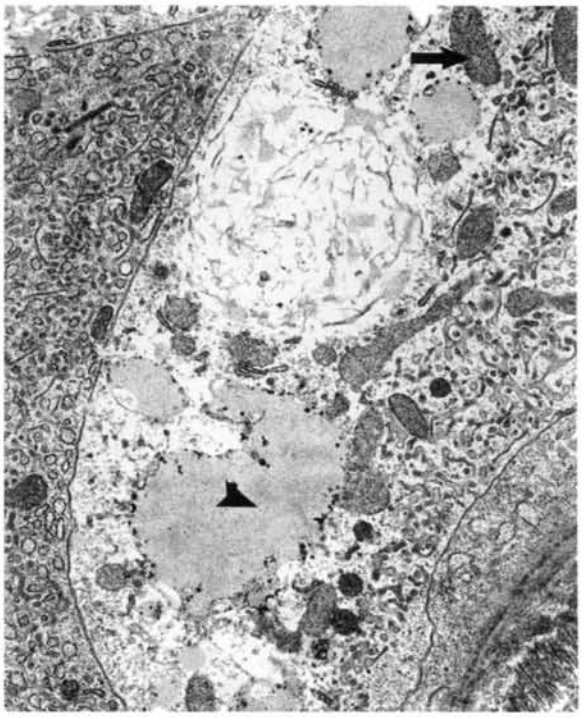


Plate 26b.Electron micrograph of hepatopancreas of shrimp given 2000 ppb aflatoxin B_1 at 8 weeks revealing fragmentation of endoplasmic reticulum (ERF), loss of cristae in mitochondria (arrow) vessicle formation (arrow head). 4000x

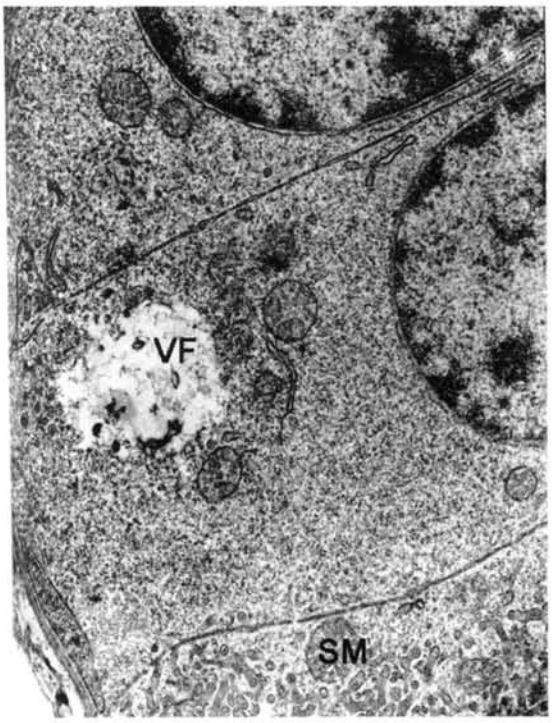


Plate 26c. Electron micrograph showing a vessicle formed (VF) in the hepatopancreas of shrimp given 2000 ppb aflatoxin B_1 at 8 weeks. Note the swollen mitochondria in the cytoplasm (SM). 5000x

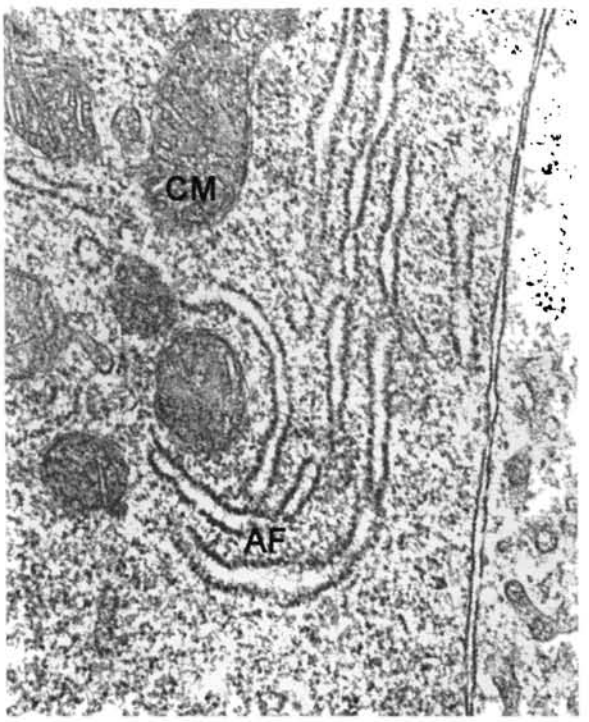


Plate 26d. Electron micrograph of hepatopancreas of shrimp fed 2000 ppb aflatoxin B_1 at 8 weeks. Note the begining of autophagy formation (AF) and condensation of mitochondria (CM). 17000x

observed (Plate 22a-22d). After 8 weeks the hepatopancreas revealed nuclear vacuolation and condensation, appearance of electron dense material, irregular shape of nucleus and loss of nuclear membrane. Fragmentation of endoplasmic reticulum was extensive There was swelling of mitochondria and vacoulation. There was loss of organelles in few areas. Formation of vesicle in the cytoplasm and loss of microvilli was observed. Cell rounding and extensive necrosis noticed (Plate 23a- 23d).

In 2000 ppb group at 4 weeks, the electron microscopic view of hepatopancreas revealed complete fragmentation of endoplasmic reticulum and degranulation. Mitochondrial damage was severe and cristae almost disappeared. Nuclear vacuolations were extensive. There was autophagic vesicle, lipid droplets and accumulation of densities in the cytoplasm. Cell membrane and microvilli were broken in many areas (Plate 24a-24d).

The hepatopancreas cells were mostly necrosed in the ultrastructural view of shrimps fed diets with 2000 ppb AFB_1 after 8 weeks. There was complete loss of cell membrane and structure of cells. Cells were completely necrosed. Microvilli were ruptured in many places with empty spaces. Mitochondria became swollen and cristae disappeared along with granules. The amount of heterochromatin in the nucleus increased. Autophagy and whorl formation were also observed (Plate 25a-25d). The rough endoplasmic reticulum lost ribosomes along with intense fragmentation of smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) (Plate 26a-26d).

4.5. Amelioration of AFB_1 toxicity by Amrita Bindu, Vitamin E and Vitamin K

In the positive control group (Group 1) and three negative control group (Group 4, 6, and 8), the survival, gross morphological features and feeding activity were normal (Plate 27a).

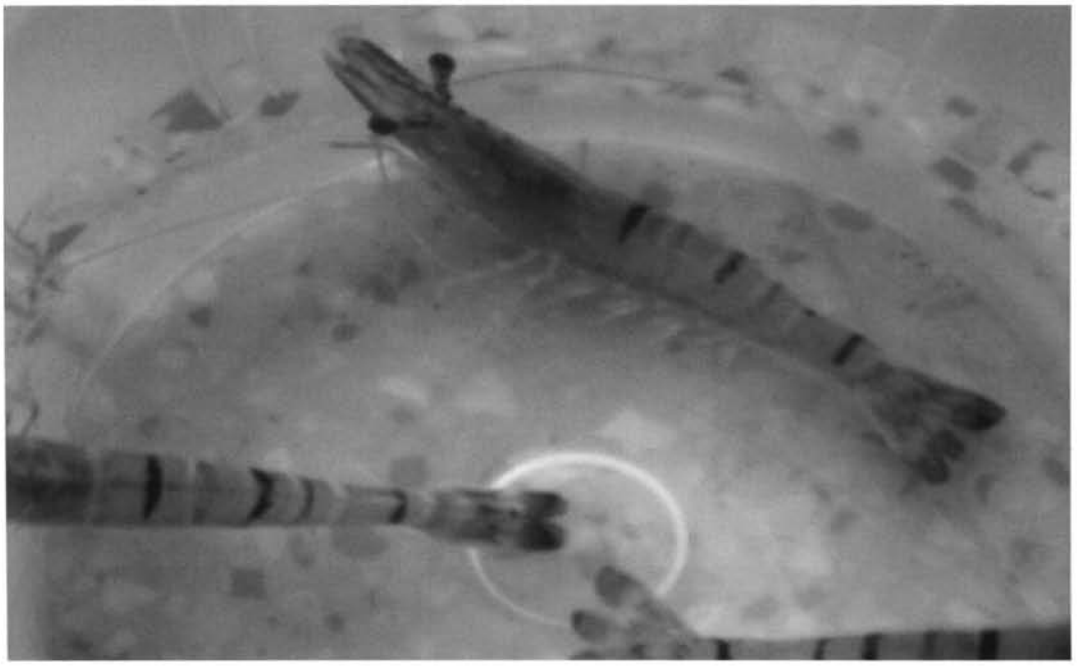


Plate 27a. *P. monodon* **of control shrimp in the amelioration study**

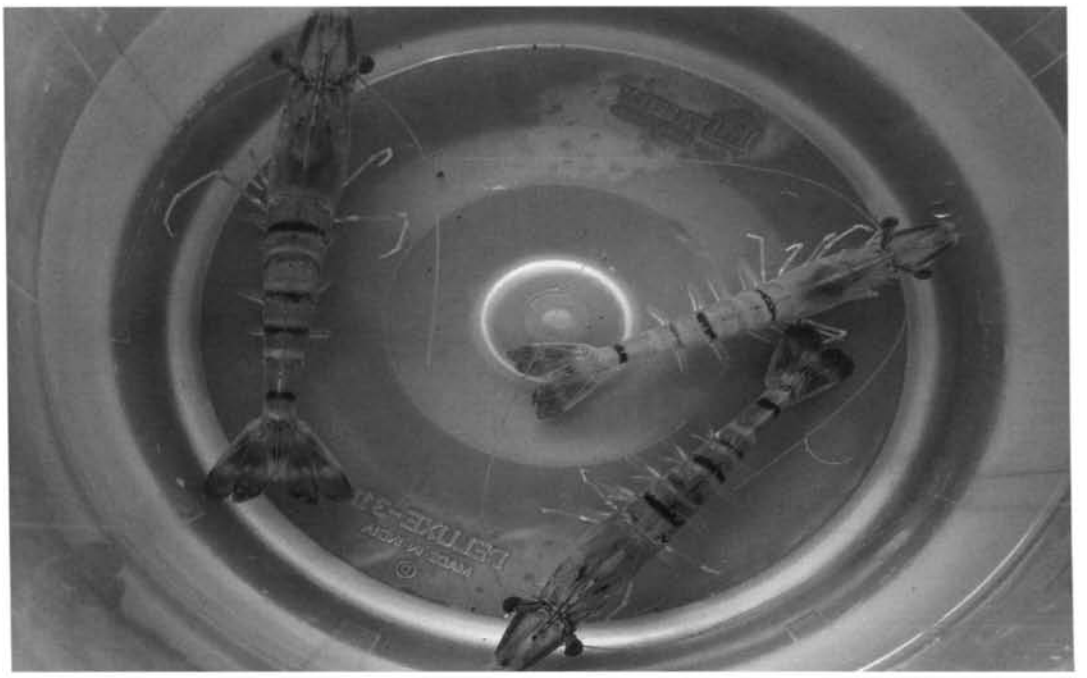


Plate 27b. *P. monodon* **fed 500 ppb aflatoxin B_1 showing reddish discolouration**

Gross pathology

After 30 days of experiment duration, there was reddish discoloration of the body (Plate 27b) and mortality in the shrimps of aflatoxin alone fed group at 500 ppb level (Group 2). Except for the discoloration there were no morphological changes in the treatment groups. In Group 3 (Amrita Bindu + AFB_1) and Group 4 (Amrita Bindu), feed intake was more and the animals were more active.

Total haemocyte count (THC)

The mean hamocyte count in the control group was $3380*10^4$ cells/ml, while in the group 2, the total haemocyte count was $2120 *10^4$ cells/ml (Table 4.43). The total haemocyte count showed no significant difference between the treatments (Table 4.44). But the total counts were observed to be relatively low in group 2 (AFB_1 fed group). While in Group 3, Group 5 and Group 7, THCs were 2924×10^4 cells/ml, 3107×10^4 cells/ml and 3359×10^4 cells/ml and observed to range between those of group 1 (control) and group 2 (AFB_1 alone fed). In group 4, 6 and group 8, the haemocyte counts were almost similar to the control group (Fig. 4.11).

Table 4.43. Total haemocyte count, acid phosphatase levels and alkaline phosphatase levels in *P. monodon* sub-adults in AFB_1 amelioration experiment

GROUPS	THC(*10^4cells/ml)	ACP(K.A.units)	ALP(K.A units)
GROUP 1	3380 ± 73.1	14.64 ±.649	$1.32 \pm.452^a$
GROUP 2	2120.8 ± 281	5.47 ±.57	5.85 ± 1.81^b
GROUP 3	2924 ± 91. 6	12.35± 2.1	2.94 ± 1.47^a
GROUP 4	4161.3 ± 712	8.48 ±.838	2.96 ± 1.73^a
GROUP 5	3101 ± 163	6.94± 1.96	1.79 ± 1.24^a
GROUP 6	3842 ± 301	9.99± 3.49	$1.89 \pm.90^a$
GROUP 7	3359.8 ± 425	5.82 ±1.28	2.99 ± 1.59^a
GROUP 8	4157.6 ± 216	8.06 ±2.09	2.46 ± 1.28^a

Values having a different superscript in a column are statistically significant.

Fig. 4.11. Total haemocyte count in *P. monodon* sub-adults in AFB_1 amelioration study

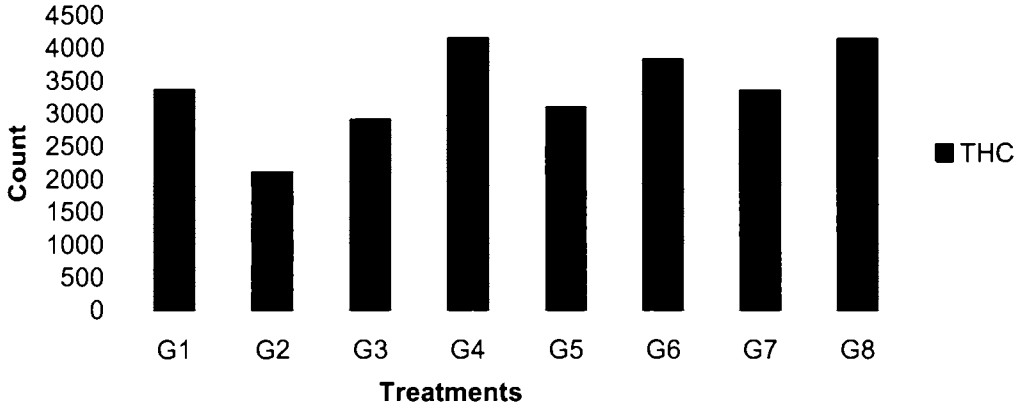


Table 4.44. ANOVA of total haemocyte count in *P. monodon* sub-adults in the AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	730.394	7	104.342	2.243, NS
Within Groups	744.235	16	46.515	
Total	1474.629	23		

NS-Not significant

Acid phosphatase (ACP)

The acid phosphatase levels were not significantly different among the treatment groups. The mean acid phosphatase (ACP) value in the control group was 14.64 K.A. units, while in the toxin alone fed group, ACP was 5.47 K.A units (Table 4.43). The ACP levels were found to be low in group 2 (AFB_1) and group 7(Vit.K+AFB_1). The mean ACP values were 12.35 K.A. units in Group 3 (Amrita Bindu +AFB_1), 6.49 K.A.units in Group 5(Vit.E + AFB1) and 5.82 K.A.units in Group 7 (Vit.K+AFB_1). While in

group 5 (Amrita Bindu), 6 (Vit.E), and 8 (Vit.K), the acid phosphatase activity was 8.48, 9.99 and 8.06 K.A. units (Table 4.45) resspectively.

Table 4.45 ANOVA of acid phosphatase activity in amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	7372466.486	7	1053209.498	.995, NS
Within Groups	16931163.189	16	1058197.699	
Total	24303629.675	23		

NS- Not Significant

Alkaline phosphatase (ALP)

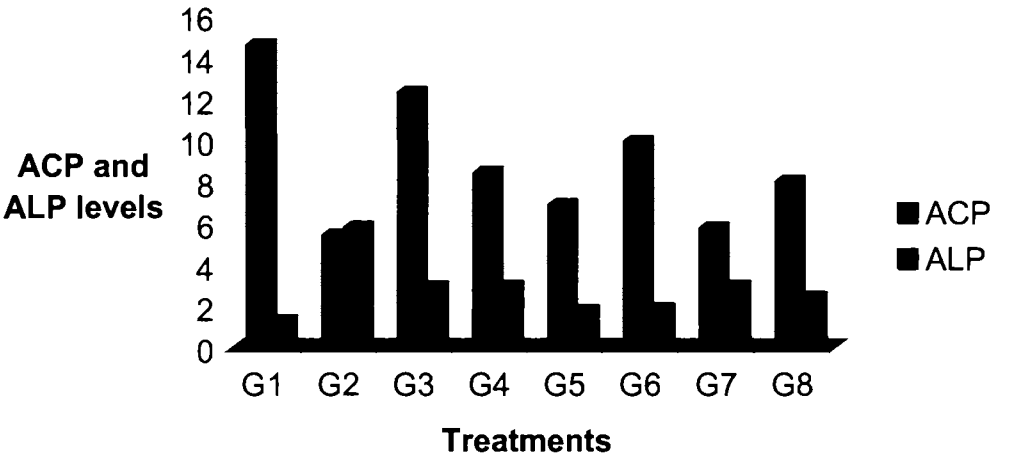
In group 2 (AFB_1), a rise in alkaline phosphatase levels was observed when compared to other groups (Table 4.43). The alkaline phosphatase levels revealed significant variation ($P<0.05$) among different groups. The ALP levels were significantly high in group 2. LSD results revealed high variation between control and group 2 (Table 4.46). There was no significant variation between other treatment groups. In the control, mean ALP was 1.32 K.A units as compared to the substantial increase of 5.85 K.A units in group 2. The alkaline phosphatase activity in other groups ranged between the levels in control and group 2 (AFB_1).

Table 4.46.ANOVA of alkaline phosphatase activity in amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	41.006	7	5.858	3.089*
Within Groups	30.346	16	1.897	
Total	71.351	23		

* Significant at ($P<0.05$)

Fig. 4.12. Acid phosphatase and alkaline phosphatase activity in amelioration study



Total protein

There was reduction in the total protein, albumin, globulin and A/G ratio in group 2 (AFB_1 group) when compared to the control (Table 4.47). The mean protein values were 9.26 g/100 ml in the control, 5.45 g/100ml in AFB_1 treatment group, 7.83g/100 ml in group 3, 8.19 in group 5, and 5.85 in group 7. The total protein levels were found to be highly significant among the different groups ($P<0.05$). Total protein levels were significantly higher in control group (Table 4.48). LSD showed maximum variation between group 1(control) and group 2 (AFB_1 group). There was no significant difference between group 2 and group 7 (Fig. 4.13).

Albumin

Albumin content was significantly ($P<0.05$) affected by the treatments. Albumin values were 1.55 and 0.87 g/100 ml in the control and toxin alone fed group. LSD results showed the same trend as in total protein (Table 4.49).

Globulin

Globulin contents were not significantly influenced by the treatments. (Table 4.50). The globulin values ranged from 4.58 g/100ml in group 2 to 7.7 g/100 ml in the group 1 (control).

A/G ratio

The mean Albumin /Globulin ratio was also not significantly affected by the treatments. The albumin – globulin ratio was relatively low in group 2 and group 5 (Fig.4.14).

Table 4.47. Total protein, albumin, globulin, albumin/globulin ratio in the amelioration experiment

GROUPS	Protein g/dl	Albumin g/dl	Globulin g/dl	A/G ratio
GROUP 1	9.26± .40^{d}	1.55 ± .06^{c}	7.70 ± .426	0.202 ± .01
GROUP 2	5.45± .81^{a}	.87 ± .19^{a}	4.58± 0.85	0.168 ± .027
GROUP 3	7.83± 1.12^{bc}	1.24 ± .106^{b}	6.13± 1.26	0.214 ± .07
GROUP 4	7.8 ±1.5^{cd}	1.24± .10^{b}	6.55 ±1.57	0.199 ± .06
GROUP 5	8.19± .65^{cd}	1.27± .21^{b}	6.92 ±.56	0.183 ± .03
GROUP 6	7.39 ±.93^{bc}	1.41± .03^{bc}	5.98 ±.95	0.24 ± .04
GROUP 7	5.85± .31^{ab}	.97 ±.136^{a}	4.87± .38	0.201 ± .04
GROUP 8	6.93 ±.61^{abc}	1.25 .105^{b}	5.67± .51	0.22 ±.01

Values having a different superscript in a column are statistically significant

Fig.4.13. Total Protein, Albumin and Globulin levels in *P. monodon* sub-adults in amelioration study

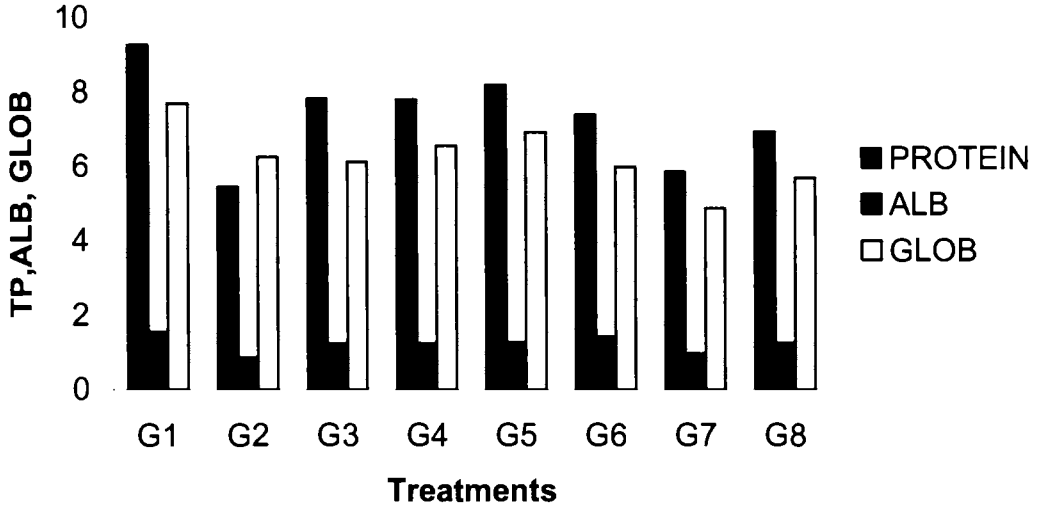


Table 4.48. ANOVA of total protein in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	31.602	7	4.515	5.900**
Within Groups	12.242	16	0.765	
Total	43.844	23		

* *Significant at (P<0.01)

Table 4.49. ANOVA of total albumin levels in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	1.009	7	0.144	7.654**
Within Groups	0.301	16	1.884E-02	
Total	1.311	23		

**Significant at (P<0.01)

Table 4.50. ANOVA of total globulin level in AFB_1 amelioration experiment

Source	Sum of Squares	df	Mean Square	F
Between Groups	14.868	7	2.124	1.639 NS
Within Groups	20.738	16	1.296	
Total	35.607	23		

NS- Not significant

Fig. 4.14. Albumin/Globulin ratio in AFB_1 amelioration study

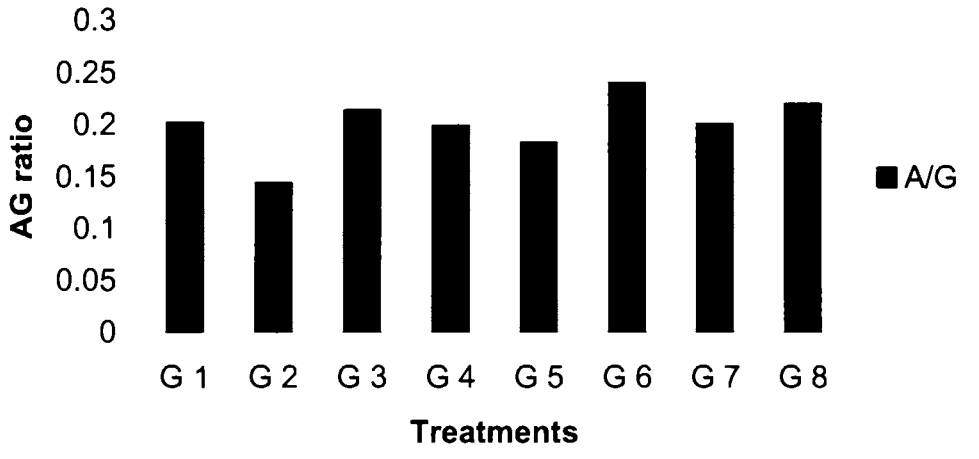
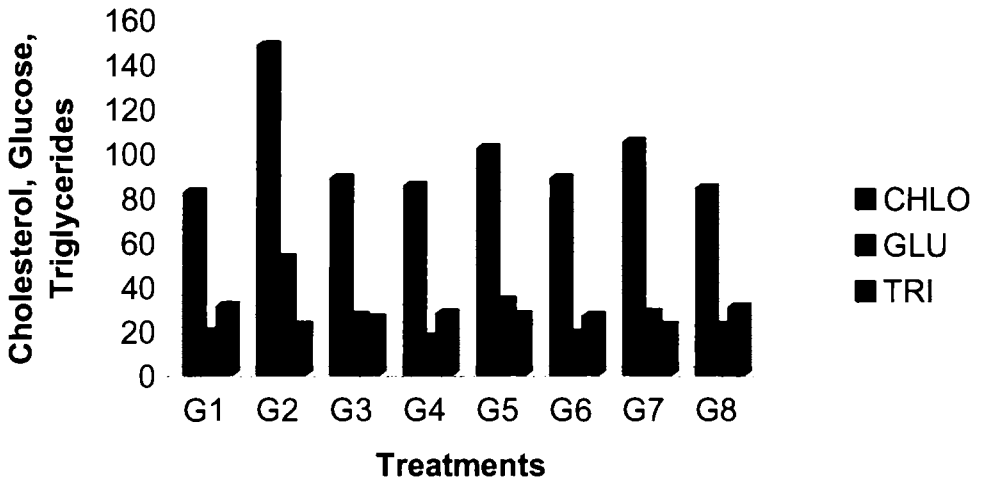


Table 4.51. Levels of glucose, cholesterol, triglycerides and lactate dehydrogenase in *P. monodon* sub-adults in AFB_1 amelioration experiment

GROUPS	Glucose mg/100 ml	Cholesterol mg/100 ml	Triglycerides mg/100 ml	LDH U/L
GROUP 1	18.83 ±1.09^{a}	81.68 ±2.98^{a}	30.61 ±1.87^{b}	629 ±28.6^{bc}
GROUP 2	52.07± 1.9^{e}	147.54± 6.3^{c}	21.7 ±3.25^{a}	1038.4 ±57.7^{e}
GROUP 3	25.84 ±4.27^{bc}	87.85± 2.1^{a}	24.72 ±2.5^{ab}	692.1± 42.8^{c}
GROUP 4	16.09 ±2.10^{a}	84.53 ±4.86^{a}	27.12 ±2.05 ^{ab}	543.6 ±33.7^{ab}
GROUP 5	33.05 ±5.11^{d}	101.45± 7.08^{b}	26.46± 1.19 ^{ab}	613.02± 8.65^{bc}
GROUP 6	17.98 ±1.8^{ab}	88.02 ±5.24^{a}	25.9± 6.4 ^{ab}	599.6± 17.6^{b}
GROUP 7	27.46± 4.59^{c}	104.32± 5.9^{b}	21.6± 1.24^{a}	919.8± 33.1^{d}
GROUP 8	21.56 ±2.06^{ab}	83.6 ±7.17^{a}	30.07± 3.05^{b}	477.7 ±95.3^{a}

Values having a different superscripts in a column are statistically significant

Fig.4.15. Cholesterol, Glucose and Triglyceride levels in AFB_1 amelioration study



Glucose

The serum glucose levels (table 4.51) showed high level of significance. ($P<0.05$). The mean glucose values were 18.83 mg/100 ml in the control group, 52.07 mg/100 ml in group 2, 25.84 mg/100 ml in group 3, 33.05 mg/100 ml in group 5 and 27.46 mg/100 ml in group 7. The mean glucose levels in group 2(AFB_1) was significantly ($P<0.05$) higher than rest of the groups (Table 4.52). LSD results revealed that group 2 and group 4 showed more variation than other treatment groups. Group 4, 6, 1 and 8 did not show much variation.

Table 4.52. ANOVA of glucose levels in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	2886.916	7	412.417	39.911**
Within Groups	165.334	16	10.333	
Total	3052.250	23		

* *Significant at ($P<0.01$)

Cholesterol

ANOVA of cholesterol levels was found to be significant (P<0.05) among the different treatments. The mean cholesterol values were 81.68 mg/ 100 ml in the control, increased to 147.5 mg/ 100 ml in group 2 (Table 4.51). The cholesterol levels were significantly (P<0.05) higher in group 2. (Table 4.53). LSD results revealed highly significant difference between control and group 2 (Fig. 4.15).

Table 4.53. ANOVA of cholesterol levels in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	10081.902	7	1440.272	47.498**
Within Groups	485.161	16	30.323	
Total	10567.063	23		

* *Significant at (P<0.01)

Triglycerides

The mean triglyceride levels (Table 4.51) were significantly (P<0.05) affected by the treatments. The mean triglyceride levels were 30.61 mg/100 ml in control, 21.7 mg/100 ml in group 2, 24.7 mg/100 ml in group 3, 26.47 mg/100 ml in group 5 and 21.6 mg/100 ml in group 7.The triglyceride levels were significantly lower in group 7 and showed more variation with the control group (Table 4.54).

Table 4.54. ANOVA of triglyceride levels in amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	235.930	7	33.704	3.441*
Within Groups	156.705	16	9.794	
Total	392.635	23		

* *Significant at (P<0.01)

Lactate dehydrogenase (LDH)

The mean LDH values and were 629 U/L in group 1 and 1038 U/L in group 2, 692 U/L in group 3, 613 U/L in group 5 and 919 U/L in group 7 (Table 4.51). High levels of LDH were observed in group 2 and group 7, while normal levels were observed in control groups. LDH levels were found to be highly significant (P<0.05) among treatments (Table 4.55) Post-hoc comparisons revealed that lactate dehydrogenase levels were significantly higher in group 2 and group 2 and group 8 (Fig. 4.16).

Fig .4.16. Lactate dehydrogenase activity in AFB_1 amelioration study

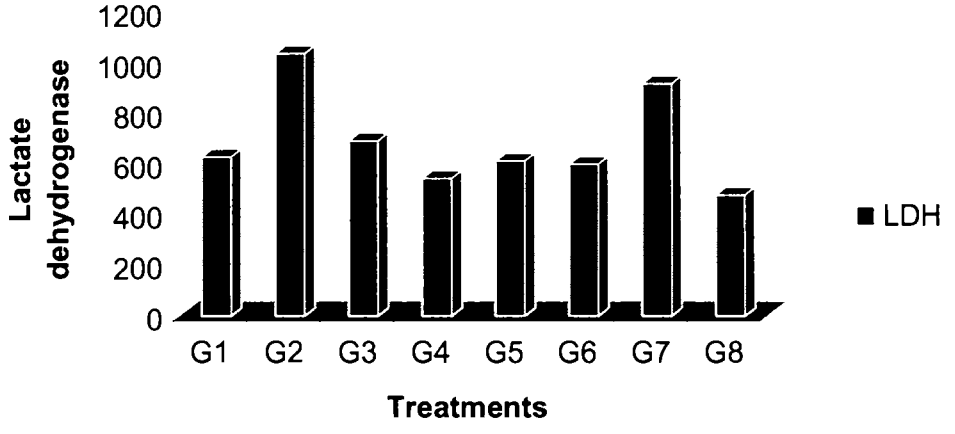


Table 4.55. ANOVA of lactate dehydrogenase activity in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	775524.256	7	110789.179	50.107**
Within Groups	35376.786	16	2211.049	
Total	810901.041	23		

* *Significant at (P<0.01)

Aspartate transaminase (AST/SGOT)

The mean values of aspartate transaminase and were 52 units/ml for control group and 80-units/ml group 2 (Table 4.56). 58 units/ml in group 3, 63.6 units/ml in group 5 and 62.66 units /ml in group 7. AST levels were significantly ($P<0.05$) higher in group 2.(Table 4.57).

Table 4.56. Aspartate transaminase, alanine transaminse activities and AFB_1 residue in amelioration experiment

GROUPS	AST units/ml	ALT units/ml	Residue ppb
GROUP 1	52 ± 6.55^{ab}	43.33 ± 4.72^{bcd}	0
GROUP 2	80 ± 1.52^{d}	63 ± 2^{e}	52
GROUP 3	58 ± 2.6^{bc}	42.33 ± 2.5^{bc}	23
GROUP 4	58 ± 4^{bc}	38 ± 4^{b}	0
GROUP 5	63.6 ± 9.6^{c}	46.33 ± 2.5^{cd}	26
GROUP 6	44.66 ± 8.5^{a}	30 ± 4.58^{a}	0
GROUP 7	62.66 ± 2.5^{c}	51 ± 3^{d}	35
GROUP 8	51.66 ± 2.51^{ab}	29.66 ± 7.6^{a}	0

Values having a different superscript in a column are statistically significant

Table 4.57 ANOVA of aspartate transaminase activitiy in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	2401.292	7	343.042	11.596**
Within Groups	473.333	16	29.583	
Total	2874.625	23		

* *Significant at ($P<0.01$)

Alanine transaminase (ALT/SGPT)

The mean values of alanine transaminase were 43.33 units/ml for control and were 63 units/ml in the toxin group (Table 4.56) Alanine transaminase levels were significantly ($P<0.05$) affected by the treatments. ALT levels were significantly higher in group 2 (Table 4.58).

Table 4.58. ANOVA of alanine transaminase activities in AFB_1 amelioration experiment

Source of variation	Sum of Squares	df	Mean Square	F
Between Groups	2542.292	7	363.185	20.271**
Within Groups	286.667	16	17.917	
Total	2828.958	23		

* *Significant at ($P<0.01$)

Residue

AFB_1 residue in carcass (Table 4.56) showed significant AFB_1 detoxification in group 3, 5 and 7. In group 2 the AFB_1 residue was 52 ppb while in group 3,5 and 7 the residue observed was 23 ppb, 26 ppb and 35 ppb respectively.

Histology

Normal structure of the hepatopancreas was revealed in the control group (Plate 28a). In group 2, hepatopancreas showed severe necrosis, detachment, and rounding of cells (Apoptosis) and shrinkage of cells. R cells were vacuolated. Fibrosis was initiated in some areas around the cells (Plate 28e-f). In group 3, there was slight necrosis in the hepatopancreas. There was thickening of tubules and detachment of cells (Plate 28b) .In group 5, there was mild necrosis in the hepatopancreas. There was slight

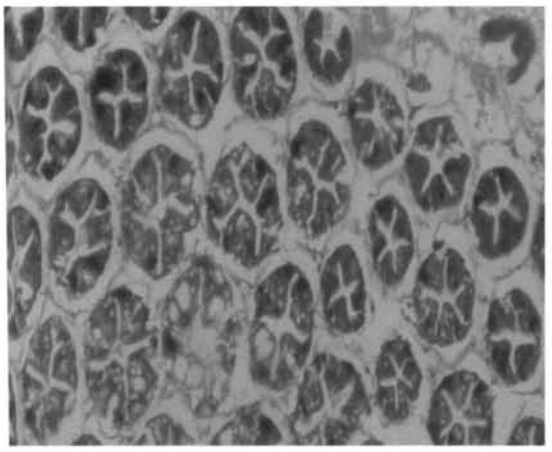


Plate 28a. Hepatopancreas section of the control shrimp used in the amelioration study. H&E 100x

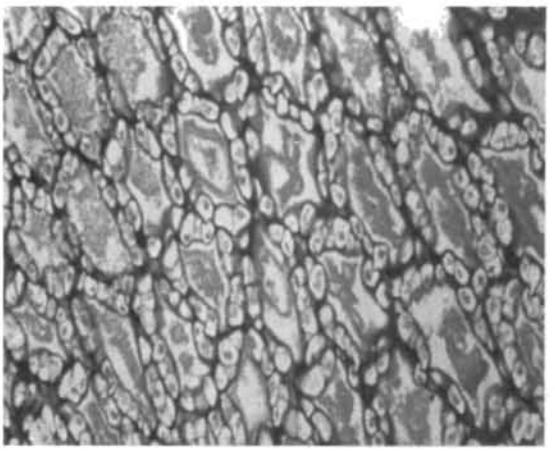


Plate 28b. Hepatopancreas section of shrimp administered Amrita Bindu for amelioration of AFB_1 toxicity. H&E 200x

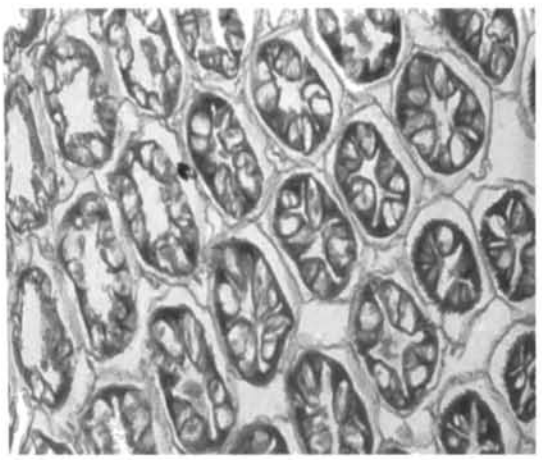


Plate 28c. Hepatopancreas section of shrimp given Vitamin E for amelioration of aflatoxin B_1 toxicity. H&E 200x

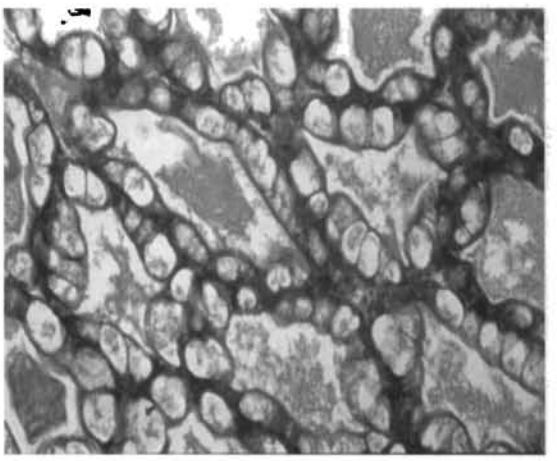


Plate 28d. Section of hepatopancreas of shrimp given Vitamin K in amelioration study. H&E 400 x

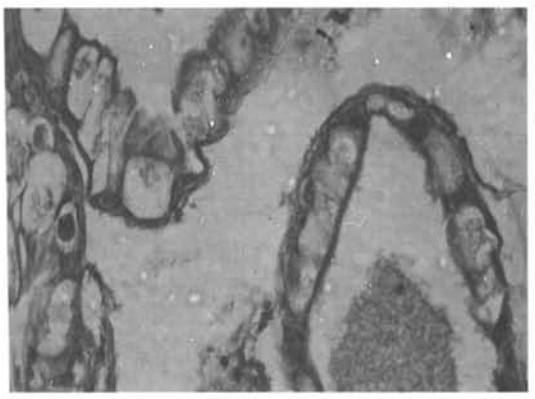


Plate 28e. Section of hepatopancreas of shrimp in group given 500 ppb aflatoxin B_1 in amelioration study revealing detachment of tubules and fibrosis. H&E 400x

Plate 28f. Hepatopancreas of shrimp in group given 500 ppb aflatoxin B_1 showing haemocytic infilittration and complete necrosed stage. H&E 400 x

detachment and rounding of cells in some areas (Plate 28c). In group 7, necrosis and fibrosis was noticed in some areas (Plate 28d).

Statistical analysis revealed significant difference in most of the parameters, between control and treatments. From DMRT, it was evident that in most of the parameters Group 3 and group 5 formed homogenous sets with the control. Group 3 appeared superior to group 5 in responding to amelioration of aflatoxin toxicity. However in most of tests, group 7 formed subset with group 2, hence the treatment with Vitamin K treatment was not found to be efficient in reducing aflatoxin toxicity.

Chapter V

Discussion

5. DISCUSSION

Aflatoxin B_1 represents by far the most toxic of all the mycotoxins, and almost all the information available on the bioactivity of aflatoxin in animals has been focused on AFB_1 and its metabolites. AFB_1 has been given prime importance due to its extreme acute and chronic toxicity, and its carcinogenic activity in animals, in addition to its potential effects in humans (Sharma and Salunkhe, 1991). Aflatoxin contamination is a problem encountered by animal feed producers and raw material suppliers especially in the humid tropical countries.

The effect of AFB_1 on *P. monodon* and its amelioration was studied considering the importance of the species in shrimp aquaculture and the limited information available in shrimps.

5.1. AFB_1 contamination in feeds and feed ingredients

The survey of shrimp and fish farms in Ernakulam District shows that feed ingredients and feeds obtained freshly from farm and stored under proper conditions contained only permissible levels of residues. The AFB_1 content increased, as the storage time prolonged and fungal growth appeared as observed in the present study. Aflatoxins have been found in feedstuffs in the United States at levels exceeding 2000 ppb, which greatly exceeds the maximum level of 20 ppb allowed in commercial feedstuffs by the U.S. Food and Drug Administration (Diener *et al.,* 1985). The survey of literature on aflatoxin levels in feed ingredients and feeds as presented in the Table 5.1 shows the presence of aflatoxin at levels much higher than the safe levels in some of the feed and ingredients reported from different countries.

Table 5.1. Aflatoxin levels in different feeds and feed ingredients

No.	Substrates	Country	AFB_1 range	Source
1.	Shrimp feeds	United States	70 ppb	Wiseman *et al.* (1982)
2.	Shrimps feeds	India	10-130 ppb	Kalaimani *et al.* (1998)
3.	Shrimp feeds	Thailand	48 ppb	Boonyaratpalin *et al.*, (2000)
4.	Shrimp feeds	Thailand	0.003-0.65 ppb	Bintvihok *et al.* (2003)
5.	Shrimp feeds and ingredients	India	Traces-250 ppb	Present study
6.	Fish feeds	India	2-100 ppm	Verma (2001)
7.	Feed samples	India	412 ppb	Dutta and Das (2001)
8.	Poultry feeds and feed ingredients	France	Traces-22 ng/g	Bauduret (1990)
9.	Poultry feed	Brazil	15-374 ppb	Maia *et al.* (2002)
10.	Corn and animal feeds	United States	2000 ppb	Diener *et al.* (1985)
11.	Corn for wild life feed	United States	Traces-750 ppb	Fischer *et al.* (1995)
12.	Peanut meal	Poland	2- 750 ppb	Strzelecki *et al.* (1988)
13.	Mustard products	India	87-1420 ppb	Sahay and Prasad (1990)
14.	Nuts, peanuts and corn	Qatar	20- 289 ppb	Abdulkadar *et al.* (2002)
15.	Oats, rye, wheat, barley, and maize	Poland	1140 ppb	Juszkiewiez *et al.* (1992)
16.	Corn samples	Panama	1290 ppb	Rojas *et al.* (2000)
17.	Corn	Brazil	0.2 – 129 ppb	Vargas *et al.* (2001)
18.	Corn flakes	Egypt	6- 10 ppm	El-Sayeed *et al.* (2003)
19.	Rice samples	Korea	4.8 ng/g	Park *et al.* (2004)
20.	Maize	Kenya	0- 58,000 ppb	Muture and Ogana (2005)

Reports from Southeast Asia convincingly prove the occurrence of aflatoxin in some fish products too. In a survey of Thailand foods, aflatoxin was detected in 5 percent of the dried fish samples at an average concentration of 166 ng/kg (FAO, 1979). In Indonesia, samples from salty fish contained AFB_1 at an average level of AFB_1 as 5ng/kg (Shank *et al.,* 1972).

The high levels of AFB_1 present in the feeds and foods emphasis the need for proper storage of these materials. Further research is also essential to elucidate the possible effects of AFB_1 on the consumers of such products contaminated with AFB_1.

5. 2. Effect of AFB_1 on growth and feed utilization in *P. monodon*

The present study on *P. monodon* postlarvae shows that aflatoxin B_1 significantly affects the growth, feed conversion, survival, protein efficiency ratio, net protein utilization and also the digestibility of feed nutrients and that the response is dose dependent as there was progressive reduction in all the response parameters with the increase in dietary AFB_1 levels. The study also reveals that the weight gain is inversely related to the AFB_1 levels in the diets. The decrease in weight gain in the AFB_1 treated shrimp groups is possibly due to the poor feed intake in 50 ppb, 500 ppb and 2000 ppb AFB_1 treatments. Poor feed intake could be due to change in palatability and decrease in metabolism caused by damage to the hepatopancreas as revealed by the histological changes.

The first two feeding trials on growth and feed performances in *P. monodon* postlarvae revealed that AFB_1 levels in the range 50 ppb - 2500 ppb in the diets of *P. monodon* could bring about changes in growth performance and histological architecture without causing complete mortality in the postlarvae. Hence the third experiment was conducted to examine the effects of

AFB_1 on other growth indices like protein efficiency ratio, net protein utilization and digestibility.

The results of these two experiments further convincingly proved the significant effect of AFB_1 in affecting growth. The reduction in growth rate is due to the poor feed conversion efficiency, which in turn reflects the poor digestibility of nutrients and reduced protein assimilation and retention as revealed by the experimental results. The low feed conversion efficiency clearly reveals that AFB_1 severely affects the digestive and metabolic processes so that the ingested food and protein are poorly converted into tissue.

Reduction in growth rate and weight gain due to AFB_1 has been earlier reported by Boonyaratpalin *et al.* (2001) in *P. monodon*. Ostrowski - Meissner *et al.* (1995) have shown that growth, feed conversion and digestibility were all affected in *P. vannamei* after 8 weeks of exposure to AFB_1. As explained by Ostrowski-Meissner *et al.* (1995) decrease in feed intake could be due to atrophy of the hepatopancreas and reduced palatability. Nile tilapia fed semi-purified diets containing 0.25 to 100mg/kg AFB_1 for 8 weeks had also reduced weight gain and hematocrit values (Anh Tuan *et al.*, 2002).

Wogan and Newberne (1967), Halver (1976) and Hendricks *et al.* (1980) have opined that the effects of aflatoxin on feed conversion ratio, weight gain and general condition of the animals was observed only after long periods of feeding very low doses of AFB_1. Jantrarotai *et al.* (1990) observed that large fingerlings of channel catfish fed sub-chronic levels of aflatoxin B_1 for 10 weeks had reduced growth rates. Chavez (1994) reported that growth rate and feed consumption were affected and histopathological changes were observed in Nile Tilapia fed different doses of toxin for 25 days.

The protein efficiency ratio (PER) is a measure of the weight gain per unit protein consumed. The reduction in PER observed in *P. monodon* could be

due to the poor protein assimilation and conversion. The apparent net protein utilization (NPU) is a measure of protein gained per unit of protein consumed by the shrimps during an experimental period. Low protein efficiency ratio and net protein utilization can be attributed to the poor digestibility of dietary protein and utilization of assimilated protein for tissue synthesis. The protein consumed could be diverted for tissue repair and defence rather than for bodybuilding. The decrease in feed and protein digestibility in aflatoxin treated groups could be due to the effect of AFB_1 on the absorptive and secretory function of the cells in the tubules of the hepatopancreas.

Reduction in specific growth rate and high values of apparent feed conversion ratio can be ascribed to the poor feed assimilation and improper absorption of nutrients due to the damage caused to the cellular architecture and organelles of the hepatopancreas. The effect of aflatoxins on protein, lipid and carbohydrate metabolism (Busby and Wogan, 1984) could be the possible reason for a reduced protein content in the tissues which reflects on the reduced weight gain and the metabolic activity of the cell. The higher mortality rates recorded in postlarvae in the aflatoxin treated groups could also be attributed to the starvation due to avoidance of toxic diets and failure of the detoxification function of the hepatopancreas.

5.3. Interaction of dietary AFB_1 with copper and cadmium in water

The interactive effect of aflatoxin B_1 in the diet and heavy metals like copper and cadmium in the rearing water was elucidated in *P.monodon* postlarvae. The combined effect of feed contaminant and environmental pollutant resulted in drastic changes in the growth performance of *P. monodon* postlarvae.

Copper and cadmium were selected for the synergistic study, as they are important heavy metals found in the estuaries and brackishwater systems along the South west coast of India and that the shrimp farms draw water from these water bodies. The levels of copper in the brackishwater systems varied from 2.72 to 17.25 ppb, while cadmium levels ranged from traces to 2.8 ppm (Anikumari, 1992). Heavy metals are stable in the environment and cannot be degraded or destroyed. Therefore, they tend to build up in the atmosphere, soils, sediments and water.

Research has shown that aquatic plants, some molluscs, crustaceans (shrimps/prawns) and fishes are not able to successfully regulate metal uptake, and as a result, tend to suffer from metal accumulation (Mohapatra and Rengarajan, 2000). Copper is an essential trace element in living organisms, and copper sulphate is commonly applied to shrimp ponds to eradicate filamentous algae and the application rate varies from 0.025 to 2 ppm (Boyd, 1990). Cadmium is one of the most harmful heavy metals to animals and has a long biological half-life and the potential has increased with increasing industrial use of this metal (Park *et al.*, 1994).

The toxicity of copper to *P.monodon* has been studied (Guo and Liu, 1992; Chen and Lin, 2001) and the 96 –hr LC_{50} of copper on juvenile *P.monodon* (0.63 ± 0.13 g) was determined as 3.13 and 7.73 ppm in seawater at 15 ppt and 25 ppt salinity (Chen and Lin, 2001). The toxicity of cadmium to *P.monodon* has also been reported (Munshi *et al.*, 1977; Sulaiman and Noor, 1996) and the 96-h LC_{50} of cadmium for *P.monodon* (PL25) was 1.7 ppm.

In the present study, since no information was available on the LC_{50} at 20 ppt salinity, the lethal doses of copper and cadmium for *P.monodon* at 20 ppt salinity were determined. The combination of sub-lethal dose of copper and 500 ppb of aflatoxin B_1 was found to be more toxic to the postlarvae than the

combined effect of sub-lethal dose of cadmium and 500 ppb of aflatoxin. The copper + AFB_1 group survived only for 24 days of the experiment while in cadmium + AFB_1 group, the cumulative effects were not severe. In copper + aflatoxin group and cadmium + aflatoxin group, there was decrease in weight gain, specific growth, feed conversion, protein efficiency ratio, net protein utilization and survival.

The current study is the first report on the synergism of aflatoxin in diet and environmental pollutants in aquatic animals. The reduction in the growth performances in the copper and cadmium treated group points out to the additive effect of aflatoxin and heavy metals. Histological study of the cephalothorax of shrimp postlarvae revealed that cumulative effects were prominent only in the hepatopancreas. The present investigation clearly revealed that aflatoxin toxicity is aggravated in the presence of heavy metal pollutant like copper and cadmium.

Further study on the interaction between sub-lethal levels of pollutants and low doses of AFB_1 is essential for a comprehensive understanding of the synergistic effect of pollutants and aflatoxins.

5.4. Pathological and immunological changes in *P.monodon* sub-adults

The results of the experiments indicate that AFB_1 severely affects the immune sysytem and hepatopancreas of *P.monodon*. By various cellular and humoral factors, the shrimps seem to respond to the fungal toxin in the circulating system.

5.4.1. Reddish discolouration

The reddish discolouration of the body in the shrimps given higher doses of AFB_1 viz, 500 ppb, 1000 ppb and 2000 ppb has direct evidence to the red disease reported in *P. styrilostris* (Lightner *et al.*, 1982), *P. vannamei* (Lightner

and Redman, 1985) and *P.monodon* (Boonyaratpalin *et al.,* 2001) given AFB_1 diets. The organ most affected due to aflatoxicosis is the hepatopancreas as evident in the biochemical and histopathological changes. As observed by Lightner *et al.* (1982), the hepatopancreas of normal decapod crustaceans contains a variety of carotenoid pigments, with most of the total body content of Beta-carotene being stored in the hepatopancreas. Atrophy and necrosis of hepatopancreas would result in release of stored beta-carotene and other carotenoids from the hepatopancreas into the haemolymph and / or a reduction of hepatopancreas uptake and storage from the haemolymph of food derived carotenoids absorbed by the midgut and hepatopancreas. Distribution and deposition of hepatopancreatic carotenoids by the haemolymph into the tissues would explain the red discolouration that characterizes the disease. Cruz and Tendencia (1989) also observed high incidence of reddening in prawns fed 150-200 ppm AFB_1 feed.

As observed in red disease in *Penaeus sp.* (Lightner *et al.*, 1982), in the current study the shrimps passed through four stages of discolouration, and finally the hepatopancreas became pale yellow in colour, lost its normal structure, and reduced to half of its original size.

5.4.2. Feed intake

Decrease in feed intake and slow growth of the sub-adults may be related to unfavourable palatability and reduced appetite caused by damage to hepatopancreas. As reported by Lightner *et al.*, (1982) lack of increased mortality may reflect a capacity of shrimps to resort to starvation rather than consume aflatoxin. Shrimps may be more likely to avoid AFB_1 contaminated feed than to consume and be poisoned by the toxin (Ostrowski-Meissner *et al.*, 1995).

5.4.3. Immune system

Aflatoxin B_1 also affected the immune functions of the shrimp. The ability of the shrimp to eliminate foreign substance is associated with haemocytes. Aflatoxins cause a decline in the activity of such immunocompetent cells and hence a decline in the shrimp's immune response (Boonyaratpalin *et al.*, 2001). Host defence is important in crustaceans, which is mainly based on haemocytes and haemolymph factors (Soderhall and Cerenius, 1992). The haemocytes play a pivotal role in the crustacean immune system and are multifunctional. They play important roles in wound repair, clotting of haemolymph, phagocytosis and encapsulation of foreign material, hardening of the cuticle and perhaps carbohydrate transport and metabolism (Soderhall and Unestam, 1979).

In the present study, the total haemocyte count, differential count, phagocytosis and phenoloxidase activity were found to increase progressively during the first four weeks with increasing concentration of AFB_1 while a reduction in these parameters was observed at 8 weeks. The high total count in shrimps fed higher levels of AFB_1 during the first four weeks might be due to increased immune response, while decrease in count after 8 weeks could be attributed to the weakening of the defence mechanism. The variation observed in the total haemocyte count is also reflected in the differential count at 4 and 8 weeks. The hyaline and semi-granular cells were found to increase at 4 weeks and decrease at 8 weeks. Phagocytosis is contributed mainly by hyalinocytes and also by semigranulocytes. Phenoloxidase is a function of semi-granulocyte and granulocytes.

The circulating haemocyte number is a stress indicator but this parameter varies non-specifically according to natural rhythms of the environment and chemical and physico-chemical stress. The decrease of

circulating haemocyte number can be a consequence of haemocyte immobilization in the gills as seen in mercury-exposed prawn (Victor *et al.*, 1990). Environmental stress can modulate the mitotic activity of haemopoietic tissues and the consequence is the reduction of the turn over of haemocytes (Johnson, 1980). Introduction of foreign substance into the circulating system stimulate the body to excrete them, which it responds by increased number of haemocytes. Owens and O'Neill (1997) observed that total haemocyte count of *P. monodon* ranged from 2100 $x10^4$ cells /ml to 2330 $x10^4$ cells /ml under normal conditions. Boonyaratpalin *et al.* (2001) reported similar findings in *P.monodon* fed AFB_1 diets for 60 days. Low haematocrit values were reported in channel catfish (Plumb *et al.*, 1986; Jantrarotai, 1990), and *Labeo rohita* (Ottinger, 1998) due to aflatoxicosis.

Differential blood cell count has long been used in the evaluation of disease processes (Mix and Sparks, 1980) and would seem to offer a potentially valuable tool in crustacean pathology. In the present study on *P.monodon*, the mean values of hyalinocytes and semi-granulocytes were found to increase at 4 weeks and decrease after 8 weeks, while granulocytes were observed to decrease at 4 weeks and increase after 8 weeks. Hyalinocytes without granules represent primitive immature haemocyte and are typically phagocytic. Semi-granulocytes, considered to be maturing forms constitute nearly 60-75% of the circulating haemocytes, are found to be involved in phagocytosis and encapsulation (Hose and Martin, 1989). Granulocytes containing large number of granules represent a completely differentiated terminal cell type does overlap in their function of cytotoxicity and storage and release of prophenoloxidase system (Mix and Sparks, 1980). Many factors like starvation (Bauchua and Plaguet, 1973) animal's physiological stage, stage of

molting cycle, may influence the relative number of granulated and nongranulated haemocytes (Mix and Sparks, 1980).

Phagocytic ratio is used to indicate the percentage of haemocytes containing endocytosed yeast/ bacteria (Smith and Ratcliffe, 1978). Phagocytosis ratio showed an increase in the treatment groups after 4 weeks of AFB_1 consumption when compared to the control in *P. monodon*. Among the cellular responses in crustaceans, phagocytosis has revealed the most attention, and this process plays an important role in the removal of foreign particles from the blood (Bell and Smith, 1993). Phagocytosis and encapsulation are universal phenomena among invertebrates. Phagocytosis is a defence mechanism when the foreign agent is smaller than the haemocyte; when the intruders are too large to be engulfed by phagocytosis, several haemocytes will then collaborate by sealing off the foreign particle from circulation.

Increase in total haemocyte count was correlated with the phenoloxidase activity in blood cells, which can fluctuate depending on haemocyte count. Studies on phenoloxidase activity in crustaceans such as shrimps and crabs indicate that complex carbohydrates can induce this enzyme activity like b-glucan and peptidoglycan that constitute the cell walls of fungi and bacteria (Soderhall, 1981; Smith and Soderhall, 1983; Song and Hsieh, 1994). Several substances in the environment such as lipids, detergents and other toxins can also stimulate phenoloxidase activity (LeMoullac *et al.*, 1998). The higher phenoloxidase activity observed in *P.monodon* at 4 weeks could be due to the increased defence response towards the toxin. After 8 weeks, phenoloxidase activity suffered a decreased response possibly due to the continuous deleterious inhibitory effect of by AFB_1.

Immuno-recognition is thought to be mediated through the prophenoloxidase system, a cascade of serum proteases and prophenoloxidase

(ProPo) present in the haemocytes, which are activated by the presence of non-self molecules initiating melanisation (Sung *et al.*, 1998). Phenoloxidase can oxidise phenols into quinones that will then polymerise non-enzymatically to melanin (Nappi and Vass, 1993). Phenoloxidase on activation is normally involved in several processes, including tanning and hardening and humoral melanisation of foreign bodies. (Soderhall and Smith, 1986; Soderhall *et al.*, 1990). The effect of AFB_1 on the cellular factors as observed in the present study conforms to the reports on the depression in the total red blood cell and white blood cell count in rats on exposure to aflatoxin B_1 (Panda *et al.*, 1975) and also to the decreased immune response in chickens, mice, guinea pigs, rats, goats and trouts (Tung *et al.,* 1975; Chang and Hamilton, 1979; Reddy *et al.,* 1983; McLoughlin *et al.*, 1984 ; Raisuddin *et al.,* 1993; Sharma, 1993; Anilkumar and Rajan, 1986; Ottinger and Kaattari ,1998).

5.4.4. Biochemical changes

Aflatoxins may be considered as biosynthetic inhibitors both *in vivo* and *in vitro*, with large doses causing total inhibition of biochemical systems and lower doses affecting different metabolic systems (Ellis *et al.*, 1991). Biochemically, aflatoxins can affect energy metabolism, carbohydrate metabolism, and nucleic acid and protein metabolism (Busby and Wogan, 1984). So in the present study changes in the activity of acid and alkaline phosphatase, and total serum protein, serum glucose, serum cholesterol in *P. monodon* sub-adults fed AFB_1 incorporated diets were studied.

Phosphatases

Acid phosphatase and alkaline phosphatase are enzymes that catalyse the hydrolysis of orthophosphoric acid esters at optimum pH levels below 7.0 and above 7.0 respectively (Kind and King, 1954). Acid phosphatase activity

increased slightly in 1000 ppb and 2000 ppb groups after 8 weeks. But alkaline phosphatase levels showed significant increase in activity with increasing doses of aflatoxin B_1 both at 4 and 8 weeks of exposure.

Acid phosphatase is very important for tissue reorganization and tissue repair (Verma and Nair, 2001). Alkaline phosphatase is a hepatopancreatic enzyme that functions in detoxification and is a good indicator of stress in biological systems (Verma *et al.*, 1980). Thus the increased alkaline phosphatase activity could be due to the increased detoxification function of the hepatopancreas. The increased activity of alkaline phosphatase in the serum observed in the present study confirms the observations of Boonyaratpalin *et al.* (2001) in *P. monodon* fed 50- 2500 ppb of aflatoxin B_1 in diets for 8 weeks.

Total serum protein

The serum proteins basically belong to two major fractions, albumin and globulin. The total proteins in plasma and sera are involved chiefly in nutrition, water distribution, acid-base balance, immunity and metabolic needs (Lehninger *et al.*, 1993). Serum protein concentration can be used to monitor disease progress and general physiological status, as total protein levels tend to drop in diseased condition. Sequential total protein analysis provides quantitative evidence of disease progression. In the present study, total serum protein varied inversely with AFB_1 levels after 4 and 8 weeks.

The reduction of protein content in serum and body leads to reduction in growth and metabolic activity of the cell. Decrease in serum protein level could be correlated with severe damage of hepatocytes. Saber (1995) has reported a reduction in total protein in aflatoxin treated *Tilapia nilotica*. George (1998) and Sahoo and Mukerjee (2001) have also observed decreased total protein values in rohu treated with aflatoxin. Inhibition of protein synthesis by

mycotoxins such as AFB_1, may arise directly from inactivation of biosynthetic enzymes, or indirectly by alteration of DNA template activity, or inhibition of RNA synthesis and maturation, translation, and/or interference with amino acid transport (Hsieh,1987). Ultrastructurally, degranulation (detachment of ribosomes) from endoplasmic reticulum frequently has been reported in AFB_1-treated cells (Terao and Ueno, 1978). Such observations may arise as a result of disruptive changes, including direct damage to the endoplasmic reticulum membranes, interference with the ribosome binding sites on the membrane, interference with the ribosomal cycle, inhibition of the release of newly synthesized proteins and a suppression of mRNA synthesis (Terao and Ueno, 1978).

Serum glucose

Blood glucose appears to be a sensitive and reliable indicator of environmental stress in fishes. Glucose levels in blood and tissues may be used to indicate the toxicological significance in the aquatic environment (Melby and Altman, 1974). An increase in the level of serum glucose was seen with increasing dietary concentration of AFB_1 in *P. monodon* during the present study, which may indicate breakdown of glycogen to glucose to meet the energy needs. Several animal species, when administered AFB_1 exhibit reduced hepatic glycogen levels and elevated serum glucose levels (Kiessling, 1986). These may arise from either an inhibition of glycogenic enzymes (e.g. glycogen synthase), an inhibition of glyconeogenesis, a decrease in glucose transport into hepatocytes or an increase in the activity of enzymes metabolising glycogen precursors (e.g. glucose 6-phosphate dehydrogenase) (Kiessling, 1986; Hsieh, 1987).

Total serum cholesterol

Cholesterol is an important precursor for steroid hormones that are essential for homeostasis, normal reproduction, moulting and response to stressful situation (Lehninger *et al.*, 1993). Cholesterol levels declined when the shrimp were given feed with higher levels of aflatoxin B_1. This can be explained by the hepatotoxic effect of aflatoxin that influences the ability of hepatopancreas to accumulate food substances, and hence results in the lowering of cholesterol. Aflatoxins were also found to affect the lipid transport and biosynthesis in chickens (Tung *et al.,* 1972) and rats (Chou and Marth, 1975)

5.4.5. Aflatoxin residues

The problem of aflatoxins doesn't end in the feed or in reduced animal performance, but remain as residues and transferred into meat, visceral organs, milk and eggs. Though the concentration of the toxins are usually lower than the levels present in the feed consumed and unlikely to cause any acute toxicity in humans, there is increasing concern about the toxic residues and their metabolites. In the present study, aflatoxin residue was detected in various tissues of the shrimp body. However no residue was detected in the control, 50 ppb, 100 ppb and 150 ppb AFB_1 treated shrimps. Residue was present in 500 ppb, 1000 ppb and 2000 ppb aflatoxin dosed shrimps after 8 weeks. According to Boonyaratpalin *et al.* (2000) no residues were detected in *P. monodon* fed 0-220 ppb toxin for 60 days. While in a subsequent study conducted in *P. monodon* fed 0- 2.5 ppm for 60 days, aflatoxin residues were dectected (Boonyaratpalin *et al.,* 2001) and the residues were highest in muscle after 4 weeks of feeding. The residues in their study decreased gradually after 6

weeks. However, the authors did not explain the reasons for the differences observed in their two studies. Jacobson (1978) detected 1-4 ppb AFB_1 residues in pigs fed 400 ppb for 4 weeks in liver, kidney, muscle and muscle tissues.

5.4.6. Histology

The histological analysis of the cephalothorax region of the control shrimps conformed to the structure described by Bell and Lightner (1988). Histological study of the cephalothorax region of the AFB_1 treatment groups revealed progressive damage to the hepatopancreas, lymphoid organ and antennal gland with increasing concentration of aflatoxin B_1. The midgut gland or hepatopancreas is considered to be the central organ of digestion in Crustacea. It is a system of blind ending tubules consisting of four cell types (Loizzi, 1971). The E cells at the summit of the tubules develop into R cells (absorption and storage of nutrients), F cells (production of digestive enzymes) and B cells (presumed to be secretory in function). The histological study of postlarvae exposed to 50 ppb AFB_1 revealed decrease in number of R cells, B cells and F cells. In the 500 ppb treatment group, there was complete loss of cells and fibrous tissue was noticed in most areas and in postlarvae treated with 2000 ppb AFB_1, the hepatopancreas revealed extensive fibrosis, haemocytic infiltration, cellular inflammation, and severe necrosis. Thus the histological analysis clearly revealed the disruption of digestive functions of hepatopancreas by AFB_1. The disruptions would upset the function of absorption and storage of nutrients due to reduced number of R cells and decrease in the production of digestive enzymes by F cells and secretion of enzymes by the B cells culminating in the disruption of digestive, metabolic and detoxification functions of the hepatopanceas.

Besides, the effect of aflatoxins on the hepatopancreas appears to be directly correlated with the concentration of aflatoxins and the duration of feeding. The experimental study clearly shows that high doses of AFB_1 are detrimental to the shrimps as the changes in the hepatopancreas were severe and intense like complete fragmentation, apoptosis, inflammation and desquamation. Smaller doses of the toxin (50ppb and 100ppb) resulted onset of necrosis and fibrosis around the tubules of the hepatopancreas and slight necrosis in the antennal gland. It is evident that even with mild doses of AFB_1, the hepatopancreas gets damaged. Histologically, aflatoxin directly attacks hepatopancreas, the main organ for detoxification of xenobiotics and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of a biological endpoint of contaminant exposure. Consequently, hepatopancreas has attracted the most attention as a target organ for biological effects monitoring programmes since pathological alterations occur at very early stage of exposure.

The first sign of toxicity observed in the present investigation was the atrophy of hepatopancreatic tubules, followed by destruction of E, R and B cells, desquamation, cellular inflammation, papillomatous growth, apoptosis in few areas, necrosis and infiltration of fibroblastic tissue between the tubules of the hepatopancreas. Only mild necrosis was observed in the antennal gland, gills and lymphoid organ. Similar changes were reported in penaeid shrimps fed AFB_1 by Lavilla-Pitogo *et al.* (1994) and Boonyaratpalin *et al.* (2001). As observed by Boonyaratpalin *et al.* (2001), AFB_1 levels above 100 ppb caused inflammation, necrosis, severe degeneration of tubules and infiltration of haemocytes. By the end of 4 weeks there were histological changes in the hepatopancreatic tissues in all the shrimp fed over 50 ppb AFB_1. In shrimps given 150 ppb and 500 ppb, the distinct changes were necrotic cells in the

lumen, loss of architecture of cells and tubules, extensive necrosis and inflammatory reaction in between tubules. In 1000 ppb and 2000 ppb groups, cyst-like and papillomatous growth was observed along with severe necrosis, cell elongation inflammatory cells, cystic hyperplasia, haemocytic infiltration, complete loss of architecture of tubules, rounding of cells or apoptosis, desquamation and cellular inflammatory response. Lightner *et al.* (1982) noted that the smallest dosage of 2 ppm in *P. stylirostris* and *P. vannamei* resulted in just detectable hepatopancreatic lesion and doses higher than 2 ppm resulted in distinctive histopathologic alterations in hepatopancreas and mandibular organs.

In *P. monodon* fed 26.5-202.8 ppb AFB_1 for 60 days the first response was in hepatopancreas and atrophy of R cells (Lavilla-Pitogo *et al.*, 1994). Penaeid shrimps fed 50-300 ppm AFB_1 showed primary lesions in the hepatopancreas, mandibular organ and haemopoeitic organs (Wiseman *et al* .,1982). In the present study the dose ranging from 50 ppb –2000 ppb were much lower than the LD_{50} value of AFB_1 reported for *P. stylirostris* and *P. vannamei* (Lightner *et al.*, 1982) hence the histological changes were targeted mainly on the hepatopancreas, while mild necrosis was noticed in the mandibular organ, lymphoid organ, antennal gland and haemopoietic organ.

According to Ostrowski-Meissner *et al.* (1995) aflatoxin related histopathologies were apparent in the hepatopancreas and antennal gland, when experimental diets containing 0-15 ppm AFB_1 were given to juvenile *P. vannamei* for 8 weeks. In contrast to the findings of Boonyaratpalin *et al.*, (2001) that no histological change were noted in *P.monodon* fed 50 ppb for 4 weeks, in the present study shrimps fed the 50 ppb AFB_1 diet at 4 weeks revealed mild necrosis and change in structure of cells and tubules. The general histopathological changes observed in the study are consistent with the

previous findings in penaeids by Lightner *et al.* (1982); Wiseman *et al.* (1982); Lavilla-Pitogo *et al.* (1994); Ostrowski-Meissner *et al.* (1995); Boonyaratpalin *et al.* (2001). Jantrarotai and Lovell (1990), and Jantrarotai *et al.* (1990) have reported necrotic foci in livers of channel catfish due to acute and subacute aflatoxicosis. Aflatoxin produced fatty liver, nuclear hypertrophy, cellular atrophy and leucocytic infiltration in the liver of *Oreochromis niloticus* (Chavez *et al.*, 1994)

5.4.7. Ultrastructure

Toxicants are known are to affect the structure and functions of cellular components leading to impairment of vital functions of many marine organisms (Baticados *et al.,* 1987). The electron microscopic view of the hepatopancreas section in the control shrimps revealed normal structure of different cells and cell organelles as described by Vogt *et al.* (1985).

The peculiar features of the ultrastructural changes in hepatopancreas of shrimps in AFB_1 treated groups were intense fragmentation of smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER), chromatin condensation, electron dense inclusions, broken cell membrane, change in shapes of mitochondria and structure of the cristae, loss of microvilli and extensive vacuolation, cell rounding, extensive necrosis, autophagy and whorl formation. Thus AFB_1 severely affected all the cellular organelles of hepatopancreas like microvillus border, cell membrane, endoplasmic reticulum, mitochondria and the nucleus in *P. monodon.*

The cell membrane controls the movement into and out of the cell and in particular controls the osmotic gradients involving fluids (Thomson, 1984), hence breakage of the cell membrane as observed in the present study would affect the normal cell functions and structure. Peroxidation of unsaturated

lipids in the bio-membranes by free radicals is a common injury, which may lead to configurational changes or breakdown. The free radicals lead to degeneration of the phospholipid layers and eventually the protein components of the cell membrane (Thomson, 1984).

The ER formed of a system of membrane bound channels in the cytoplasm functions in the protein synthesis, lipid metabolism, glycogen storage, synthesis of membrane proteins on the ER membrane and its transport through ER lumen, detoxification of drugs, cell to cell communication and muscle contraction by release and uptake of calcium ions (Lehninger *et al.*, 1993). In the present study, the endoplasmic reticulum has undergone few conformational changes as a result of aflatoxin contamination and notable among them are dilations, degranulation, peroxidation and fragmentation. Dilations of endoplasmic reticulum (ER) is known to cause changes in ion and water movements, degranulation causes loss of polysomes from ER, peroxidation results in change of membrane conformities and accumuation of densities, while progressive fragmentation of ER results in smooth vesicles and detached polysomes (Thomson, 1984). Thus AFB_1 disrupts ER, and reduces the RNA synthesis, attachment of polyribososmes to ER, and damages ribosomes also, thus severely affecting protein synthesis. This could be the possible reason for the reduction in protein level in serum observed in the present study.

Mitochondria, the powerhouse of the cell is associated with generation of ATP (energy) through electron transport and oxidative phosphorylation (Lehninger *et al.*, 1993). In the current study, severe alterations in mitochondrial configuration, condensation followed by swollen nature and loss of cristae, and severe damage were observed in 2000 ppb AFB_1 group after 8 weeks. The condensed state of mitochondrion reflects the contraction of matrix

proteins following changes in ATP/ADP ratio and the rupture of outer membrane of mitochondria there is ion and water movements resulting in swelling of mitochondria, finally with the aggregation of denatured proteins the mitochondion appears severely damaged (Thomson, 1984). Mitochondrial damage and disappearance of cristae affect enzymes and electron carriers for formation of ATP and TCA cycle in turn affect cellular respiration. Thus the vital cellular functions are affected (Lehninger *et al.*, 1993).

Autophaghic vesicles were observed in the ultrastructural study of the heaptopancreas. Lysosomes are concerned with removal of degenerated components within the cell by autophagy or self eating process (Thomson, 1984). Nuclear damage observed in the current study includes nuclear vacuolation, shrinkage, and condensation. The toxic insult interacts with the complex structure of chromatins, by forming Aflatoxin-DNA adducts (Scarpelli and Trump, 1964). The nuclear damage might be the reason for decrease in the total protein content observed in the present investigation. This is further evidenced by ER degranulation. The effects on the ultrastructure of hepatopancreas due to AFB_1 are consistent with previous reports on aflatoxicosis in rainbow trout (Scarpelli *et al.*, 1963; Nunez *et al* .,1991).

5.5. Detoxification

Detoxification of aflatoxin contaminated foods and feeds is a current thrust area of research, as aflatoxins are highly carcinogenic and capable of passing unaltered through metabolic processes and accumulating in the tissues thus seriously jeopardizing human and animal health. Although numerous detoxification methods have been tested, none seems able to fulfill the efficacy,

safety, safeguarding of nutritional elements and costs requisites of a detoxification process (Piva *et al.*,1995).

The best defense against mycotoxin is the selection of "clean," mycotoxin-free feeds and feed ingredients. The best way of obtaining these ingredients and feeds is to build a good relationship with suppliers and to maintain standard operating procedures that prevent mould growth. However, this can be a challenge in the warm, wet conditions, which prevail in many aquaculture production areas, especially when feeds or raw materials are stored under poor conditions (Park, 1993; Park *et al.*, 2004).

Aflatoxins are considered unavoidable contaminants of food and feed, even where good manufacturing practices have been followed. The FDA has established specific guidelines on acceptable levels of aflatoxins in human food and animal feed by establishing action levels that allow for the removal of violative lots from commerce. The action level for human food is 20 ppb total aflatoxins. However, it is very difficult to accurately estimate aflatoxin concentration in a large quantity of material because of the variability associated with testing procedures, hence the true aflatoxins concentration in a lot cannot be determined with 100 % certainity (Park, 1993).

In the current study, Amrita Bindu, Vitamin E and Vitamin K were used for evaluating their efficacy in amelioration of aflatoxin B_1. With all the detoxification methods, the response parameters tested showed almost normal levels when compared to the aflatoxin toxin contaminated group fed 500 ppb AFB_1.

5.5.1. Total haemocyte count

The total haemocyte count decreased in the AFB_1 treated shrimp group when compared to the control group, while in the detoxifying groups, there was

slight increase in the haemocyte count. In group 3 (aflatoxin + AB), group 5 (aflatoxin +Vit. E), and group 7 (aflatoxin +Vit. K), the THC was almost comparable to the control shrimps suggesting the positive ameliorative effect of the three compounds tested. As pointed by Persson *et al.* (1987) a low circulating haemocyte number coincides with higher susceptibility to infectious diseases. Studies in vertebrates afflicted by aflatoxicosis have revealed a reduction in haemocrit values and production of anaemia (Wannop, 1961; Brown and Abrams, 1965). In Channel catfish, decrease in erythrocyte counts and anaemia were observed in aflatoxin treated fishes (Jantrarotai and Lovell, 1990).

Biochemical responses

5.5.2. Enzymes

Acid phosphatase and alkaline phosphatase are good indicators of stress in biological systems (Verma *et al.*, 1980). Acid phosphatase decreased in the toxin alone fed group after 30 days of detoxification, while alkaline phosphatase increased in the toxin group. In groups exposed to aflatoxin and ameliorating agents, the phosphatases activity ranged between those of the control and aflatoxin alone fed groups. Imbalances in the level of phosphatase in the serum could be attributed to the damage of the hepatopancreas by toxic insult.

Aspartate transaminases (AST) and Alanine transaminases (ALT) are liver enzymes, that showed significant increase in toxin alone fed group compared to the control groups. In the three-detoxification treatments with Amrita Bindu, Vitamin E and K, AST and ALT were also comparable to the control group. The histological observation also revealed damage of the hepatopancreas, which explains the increased levels of transaminases. ALT is a

cytoplasmic tramsaminase present in hepatic cells and released from cytoplasm from injured liver cells in moderate injuries, while AST is located in mitochondria and released during severe injury (Thomson, 1984). The enzymes are released into the circulating system by cellular damage or destruction (Melby and Altman, 1974). Liver is rich in AST and ALT and changes in plasma levels of these enzymes may be indicative of liver dysfunction (Kapila, 1999). Increased levels of transaminases in serum of aflatoxin dosed *P. monodon* sub-adults were similar to the findings of Boonyaratpalin *et al.* (2000) in *P. monodon*. In groups other than the aflatoxin alone fed group, transaminase levels were similar to that of the control group. Thus the elevated levels of AST and ALT could be correlated to the severe damage to the heaptopancreas in *P. monodon* exposed to AFB_1, suggesting the ameliorating effect of Amrita Bindu, Vit. E and Vit.K.

Lactate dehydrogenase (LDH) is responsible for the formation of lactic acid during glycolysis and its oxidation to pyruvate during respiration. Serum LDH is brought about by cellular damage or destruction. (Melby and Altman,1974). Normal levels found in serum are indicative of body's routine destruction of senescent cells and their replacement. Abnormally high levels result from tissue damage (Kaplan and Pesce, 1996). Increase of LDH activity in serum of toxin fed group indicated a shift towards anaerobiosis indicating that pyruvate oxidation is not favourable. LDH is a parameter widely used in toxicology and in clinical chemistry to diagnose cell, tissue and organ damage (Diamantino *et al.*, 2000). In the current study, high LDH levels were observed in toxin alone fed group and aflatoxin + vitamin K group. In other groups LDH levels were not significantly different from the control groups. Increase in LDH levels were also reported by Saber (1995) in Nile Tilapia due to aflatoxicosis.

5.5.3. Serum Glucose, Cholesterol, Triglycerides and Protein

Increase in Glucose level in the aflatoxin alone fed group may be due to reduction in the glycogen synthesizing ability of tissues as a result of cellular damage. While in the detoxification treatment groups with herbal powder and vitamins, the glucose levels were almost normal as in the control group. In all the treatment groups except the aflatoxin alone fed group, the serum glucose levels were similar to the levels in the control group, which could be due to the ameliorative action of the compounds used.

Cholesterol, phospholipids and triglycerides constitute the three large lipid fractions of the serum. Cholesterol is an important precursor for steroid hormones that are essential for homeostasis, normal reproduction and response to stressful situation. The total cholesterol level increased in the aflatoxin alone dosed group. While cholesterol levels were almost similar in the control and other treatment groups, attributing to the ameliorative effect of the herbal powder and vitamins added to the diets.

Triglycerides in the serum denote the amount of neutral fat content; a decrease in the serum level of triglycerides indicates a disorder in lipid metabolism caused by the toxin. Impaired triglyceride transport and lipid biosynthesis has been reported as a primary lesion in chickens by aflatoxin (Tung *et al.*, 1972). In the present study, serum triglycerides were low in aflatoxin alone fed group (group 2), while in other treatment groups, the levels were between those of control group and toxin alone fed group (group 2). This suggests the possible ameliorative effect of the materials incorporated in the feed.

Total protein and albumin showed significant variation in the treatment groups, while not much variation was noticed in globulin and A/G (Albumin/Globulin) ratio. Aflatoxins affect protein metabolism and

hepatopancreas, hence contributes to reduction in protein content. The reduction in serum protein and albumin could be attributed to the severe effect on the hepatopancreas. Decreased protein content has been reported for aflatoxicosis in Nile Tilapia (Saber, 1995) and rohu (George, 1998 and Sahoo and Mukherjee, 2001;), land animals and birds (Brown and Abrams, 1965; Singh *et al.,* 1987; Harvey *et al.*, 1988). The levels of protein, albumin and globulin in the groups added ameliorating agents were between those of the control and aflatoxin fed group, which directly points to the positive effect of the materials added in the diets.

5.5.4. Histology

Histological study of toxin alone fed group has revealed extensive necrosis, fibrosis and rounding of cells or apoptosis in the hepatopancreas after one month of treatment with 500 ppb AFB_1. There was only mild change in the structure of tubules of the hepatopancreas in the three groups fed the ameliorative materials, which indicates the possible amelioration of AFB_1 by the herbal powder and vitamins.

5.5.5. Amelioration by Amrita Bindu, Vitamin E and K

Detoxification by Amrita Bindu (AB) has proved to be more efficient when compared to Vitamin E and Vitamin K in the present study with 500 ppb AFB_1. From the results it is evident that AB is able to reduce AFB_1 mediated toxicity in the shrimps and reduce the toxic residues in the shrimp body by 55.76%. All the cellular and humoral factors showed almost normal levels comparable to the control group. Feed intake was more in Amrita Bindu incorporated diet groups. No morphological changes and reddish colouration was noticed in the group 3 and the behavior of the shrimps were similar to the

control group. Amrita Bindu is a herbal mixture and has been found to provide protection against nitrosoamine induced lipid peroxidation and tissue degeneration in rats. The possible mechanism of action of the mixture may be due to binding of aflatoxins and its immobilization, through silicate and plant lignin in the mixture (Shanmugasundaram *et al.*, 1993). Antioxidant and reducing power of the mix may be due to the presence of flavanoids, terpenes, phenolics in the herbs and spices that make up the extract. In the Indian carp, rohu, detoxification of AFB_1 by Amrita Bindu improved the total protein levels and other lipid peroxidation parameters (Sujatha, 1990; Shanmugasundaram *et al.*, 1993; Madhusudhanan, 2001).

Detoxification by Vitamin E was almost similar to the herbal extract. Aflatoxin residue analysis showed the residue was reduced by 50% (26 ppb). Vitamin E (a-tocopherol) functions as a lipid soluble chain antioxidant, which protects lipids, proteins and membranes from oxidative damage. a-tocopherol plays a major role in maintaining the integrity of cell membranes in general and lymphocyte membrane in particular as well as probable role in reducing free radical damage during aflatoxicosis. In addition antioxidant vitamins have high safety margins and megadoses have positive roles on the immune system (Waagbo, 1994). Sahoo and Mukherjee (2002) observed that feeding of a high level of a-tocopherol to AFB1 treated immunocompromised *Labeo rohita* significantly raised specific immunity, non-specific resistance factors and disease resistance capacity. Influence of Vitamin E on immune response in vertebrates, including several fish species was reviewed by Tengerdy *et al.* (1981), Lall (1988), Landolt (1989) and Waagbo (1994). Vitamin E, a potent biological antioxidant has a higher affinity for aflatoxin and acts by reducing its bioavailability through the formation of stable association. a-tocopherol treatment significantly ameliorates the aflatoxin-induced changes in the testis

of mice (Verma and Nair, 2001). In Vitamin E and toxin-incorporated group, the results of the observed parameters were almost normal and the hepatopancreas revealed only mild necrosis and regeneration foci in many areas.

Vitamin K is essential for normal blood clotting mechanisms and energy metabolism and the deficiency of which leads to anaemia, haemorhagic gills, eyes and vascular tissues. Cowey *et al.* (1985) have reported the ameliorative capacity of Vitamin K on aflatoxin in fishes. Menadione used in the current study however did not effectively detoxify aflatoxins. The residue retention was 35 ppb and the residue analysis revealed only 32.69% detoxification. In channel catfish, higher amounts of Vitamin K reduced the haemorrhages commonly encountered with aflatoxin intake, fishes fed 50-100 mg menadione sulphate/kg diet along with 100-300 ppb crude aflatoxin showed a rapid improvement in haemopoiesis and a return to normal haematocrit and red blood cell count with one week after Vitamin K supplementation (Cowey *et al.*, 1985).

These results indicate the usefulness of additives and antioxidants in ameliorating aflatoxin induced toxicity in *P. monodon*. It is likely that these feed additives act by inhibiting activation of aflatoxins and increasing its detoxification. Further work is essential by optimizing the ratio of the aflatoxin doses to the amelioration agent incorporated in the diet. More comprehensive research is also necessary to carry out long term feeding trials to elucidate the detoxification mechanism in shrimps in combating the toxicity of aflatoxins.

Conclusion & Summary

CONCLUSION

The current study has revealed that Aflatoxin B_1 significantly affects protein, lipid and carbohydrate metabolism in the shrimp *Penaeus monodon*. The remarkable effect was observed in the immune system, as AFB_1 has elevated the immune response during initial days of exposure and prolonged exposure to the toxin leads to weakening of the animal's immunity. Aflatoxin B_1 levels above 50 ppb severely affected the growth and feed utilization which in turn reflects the damage caused to the hepatopancreas as evident from the histological and ultrastructural observations. The dosages of AFB_1 500 ppb and above in the diet resulted in toxic residues in the shrimp carcass. The reddish discolouration of the body formed in shrimps fed 500 ppb toxin and above is a direct indication of the red disease and the dysfunction of the detoxification role of the hepatopancreas. The elevated levels of the digestive enzymes indicated that high toxin levels affect the entire metabolic functions of the animal. The toxic effect on protein metabolism is revealed by the decreased serum protein level, degranulation and fragmentation of endoplasmic reticulum, condensation of chromatin in the nucleus. Increased serum glucose levels reveal the effect on carbohydrate metabolism. The changes in serum triglycerides and cholesterol levels project the deleterious effects on the lipid metabolism. The toxicity of aflatoxins can be exhilarated by other factors in the feed or in the rearing water. The interaction of several factors like microbes, pollutants, heavy metals and fluctuations in environmental parameters can lead to synergistic effects in the cultured organisms. Amelioration of aflatoxin B_1 toxicity by natural products like vitamins and herbal products in shrimps give more scope for further research in identifying new compounds and optimum dosages.

SUMMARY

Penaeus monodon is the most widely farmed shrimp in India and elsewhere. The present study was undertaken to elucidate the nutritional and pathological changes associated with aflatoxin B_1 toxicity in *Penaeus monodon* and to determine the efficacy of vitamins E and K, and Amrita Bindu, a herbal mixture in ameliorating the toxicity of AFB_1.

A survey conducted to detect aflatoxin contamination in shrimp and fish feeds used in farms located in Ernakulam District, Kerala, India, revealed that feeds and feed ingredients obtained freshly from farm and stored under proper conditions contain only safe levels of AFB_1 (< 20 ppb), while those stored for six months had AFB_1 levels in the range of 150 - 250 ppb.

Three sets of experiments were conducted in *P. monodon* postlarvae to determine the effect of AFB_1 on growth and feed performances.

- The first experiment was conducted in postlarvae of size 0.06 ± 0.013 g to determine the effects of selected levels of AFB_1 viz., 0 ppb, 20 ppb, 50 ppb, 2500 ppb and 5000 ppb on weight gain, survival and histological architecture. This trial was conducted to determine the doses of AFB_1 to be taken for further study. The results of the experiment showed that 50 ppb to 2500 ppb AFB_1 could produce chronic aflatoxicosis in *P. monodon.*
- The second experiment was conducted on postlarvae of size 0.56 ± 0.001 g to determine the effects of different doses of AFB_1 viz., 0 ppb, 50 ppb, 250 ppb, 500 ppb, 750 ppb, 1000 ppb and 2000 ppb on weight gain, specific growth rate, apparent feed conversion and survival rate. The trial revealed highly significant ($P<0.05$) effect of AFB_1 on the response parameters.
- The third experiment with four treatments of 0 ppb, 50 ppb, 500 ppb and 2000 ppb AFB_1 in postlarvae of size 0.11 ± 0.02 g was conducted to

determine the effects on weight gain, specific growth rate, apparent FCR, protein efficiency ratio, net protein utilization, and feed and protein digestibility.

- There was progressive increase in mortality and feed conversion ratio, with increase in AFB_1 levels in the diets; where as weight gain, specific growth rate, feed digestibility, protein digestibility, protein efficiency ratio and net protein utilization decreased as the doses of aflatoxin B_1 increased in the treatment groups.
- Weight gain and specific growth rate were significantly ($P<0.05$) affected by the AFB_1 levels in the diets. There was three-fold decrease in weight gain and 50% decrease in specific growth rate in 2000 ppb group when compared to the control group.
- Apparent feed conversion ratio showed a 60% increase in 2000 ppb group when compared to the control and the values showed a positive correlation with increasing concentration of AFB_1.
- The mean feed and protein digestibility values were significantly ($P<0.05$) lower in all the AFB_1 treatment groups when compared with that of the control group.
- The protein efficiency ratio and net protein utilization values showed a significant ($P<0.05$) decrease in 50, 500 and 2000 ppb AFB_1 groups when compared to the control group.
- Histological changes in the hepatopancreas were directly correlated with the increasing concentration of AFB_1 in diets. The prominent histological changes were atrophy of R cells, cell elongation, hyperplasia and necrosis in the hepatopancreas of the postlarvae.

The interactive effect of 500 ppb of aflatoxin B_1 in diet with sub-lethal doses of copper and cadmium in the rearing water was elucidated for 40 days in *P. monodon* postlarvae of size 0.55 ± 0.04 g.

- The LC_{50} levels of copper and cadmium to postlarvae at 20 ppt salinity were determined as 5.26 ppm of copper and 1.92 ppm of cadmium and the sub-lethal levels of copper (0.526 ppm at 20 ppt salinity) and cadmium (0.192 ppm at 20 ppt salinity) were selected for study.
- After 24 days of the experiment, there was complete mortality in copper + aflatoxin group, and the experiment was continued with the control and cadmium + AFB_1 groups. And the experiment was terminated after 40 days, when the cadmium + aflatoxin group also showed less survival,
- The weight gain, specific growth rate, apparent FCR, survival, protein efficiency ratio and net protein utilization were significantly ($P<0.05$) lower in the cadmium + aflatoxin B_1 group when compared with the control group and aflatoxin B_1 group.
- Histological changes revealed complete atrophy of hepatopancreas in copper + toxin group. Hepatopancreas of cadmium + aflatoxin group showed less severe effect when compared to the copper+ aflatoxin group.

The effects of different doses of AFB_1 (0, 50 ppb, 100 ppb, 150 ppb, 500 ppb, 1000 ppb and 2000 ppb) on pathological and immunological changes in *P. monodon* sub-adults were studied for 8 weeks. The salient findings are listed below.

- Shrimps fed 500 ppb, 1000 ppb and 2000 ppb AFB_1 diets exhibited reddish discolouration of the body.
- Poor feed intake, high mortality, slow growth rate were observed in groups fed with AFB_1 levels above 500 ppb after 4 weeks.
- The AFB_1 doses showed highly significant ($P<0.01$) effect on the total haemocyte count with an increase at 4 weeks and decrease at 8 weeks in the treatment groups when compared to the control group.

- Differential haemocyte count did not reveal any significant difference among AFB_1 treatments after 4 and 8 weeks, but hyalinocytes and semi-granulocytes increased at first 4 weeks and decreased in next 4 weeks when compared to the control group.
- Phagocytic ratio and phenoloxidase activity were found to be higher in treatment groups at 4 weeks, while decreased at 8 weeks as compared to the control.
- Acid phosphatase levels were significantly ($P<0.01$) higher in 1000 ppb and 2000 ppb AFB_1 treated shrimp than the control.
- Alkaline phosphatase level though increased at 4 and 8 weeks in the treatment groups as compared to the control, is not statistically significant.
- Serum glucose levels were higher in the treatment groups after 4 and 8 weeks, while serum cholesterol were lower at 4 and 8 weeks in the AFB_1 treatment groups as compared to the control group. Total serum protein was significantly ($P<0.05$) lower in the treatment groups when compared to the control group.
- Aflatoxin residues were detected in groups given diets aflatoxin containing levels above 500 ppb.
- Histological changes were directly related to concentration of AFB_1 in the diets. The important changes in the heptopancreas were reduction in the number of R cells, B cells and F cells, loss of structure of cells and tubules, desquamation in the tubules, fibrosis, necrosis, cellular inflammation, haemocytic nodule formation and haemocytic infiltration. Other organs affected were gills, lymphoid organ and antennal gland.
- The remarkable features of the ultrastructural study of the hepatopancreas were rupture of cell membrane and microvillus border, swelling of mitochondria, degranulation and fragmentation of endoplasmic reticulum, formation of autophagic vesicles, accumulation of densities, nuclear vacuolation and chromatin condensation.

Amelioration of aflatoxin B_1 toxicity was attempted in sub-adults of *P. monodon* with Amrita Bindu, Vitamin E and Vitamin K. There was a positive control group, and a negative control group for each treatment. The toxin level selected was 500 ppb aflatoxin B_1 for amelioration. The response parameters observed were total haemocyte count and biochemical parameters like acid phosphatase, alkaline phosphatase, alanine transaminse, aspartate transaminase, lactate dehydrogeanse activity, levels of glucose, cholesterol, total protein, albumin, globulin, albumin–globulin ratio, triglycerides in the serum and histological changes in the hepatopancreas.

- Shrimps fed AFB_1 alone (Group 2) had reddish discolouration of the body.
- Total haemocyte count showed no significant variation between the treatments but the haemocyte count in the three groups fed with ameliorating agents was almost similar to the control group.

- The different enzyme levels taken for study in the group fed with the ameliorating agents, Amrita Bindu and Vit. E were comparable to the control group. While in group 7 (Vit. K), the levels of enzymes were comparable to aflatoxin fed group.

- The histological changes were severe necrosis in the aflatoxin alone fed group, while the section of hepatopancreas revealed mild fibrosis and necrosis in groups fed with ameliorating agents.

- Residue analysis of the shrimps from different treatment groups showed that (Amrita Bindu) and Vitamin E were more effective in ameliorating AFB1 toxicity as compared to Vitamin K.

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