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 B₁, B₂, G₁, G₂ representing their blue and green fluorescence in UV light. Two other familiar aflatoxins, M₁ and M₂ are in fact metabolites of B₁ and B₂ and labelled so because of their presence in milk of animals previously exposed to B₁ and B₂ (Stoloff, 1976). The aflatoxins display potency of toxicity and carcinogenicity in the order of AFB₁ > AFG₁ > AFB₂ > AFG₂ as illustrated by their LD₅₀ 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 haemorrhages 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 hepatotoxicity 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. Carcinogenicity

Aflatoxins are highly carcinogenic to some species such as rats (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 B₁ 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 responsible 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 B₁ 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 AFB₁ (Lee et al., 1978).

The combination of rainbow trout and AFB₁ 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 LD₅₀ dose of AFB₁, 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 AFB₁ – 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 AFB₁ 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 B₁ 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 AFB₁ 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<sub>1</sub> 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<sub>1</sub>. 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, carcinogenicity 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 AFB₁ 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 catfish (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 AFB₁ were fed (Ottinger and Kattari, 1998).

2.1.6.6. Biochemical effects

Taylor et al. (1973) observed changes in activity of liver enzymes like glucose-6-phosphate dehydrogenase, NADP-linked isocitrate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in rainbow
trout fed 20 ppb AFB₁. Juvenile rohu (200-300 g) fed 400 ppb aflatoxin B₁ 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 AFB₁ 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 LC₅₀ 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 AFB₁ 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 AFB₁ /g body wt) and *P. vannamei* of 0.5 g average weight were fed different doses of aflatoxin (53-300 μg AFB₁/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 LD₅₀ was found to be from 90 to 200 ppm, and the single dose LD₅₀ was found to be approximately 25 ppm (Lightner *et al.*, 1982).

Wiseman *et al.* (1982) studied the toxicity of AFB₁ in *P. stylirostris* by intramuscular injection and found that the 24h and 96h LD₅₀ 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 B₁ contaminated diets (26.5 to 202.8 \( \mu g/kg \) AFB₁ for 60 days). Shrimp fed diets with more than 50 ppb AFB₁ exhibited 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 AFB₁, 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 AFB₁ 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 AFB₁ (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 B₁ 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 B₁ for 3 weeks showed that AFB₁ 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 B₁ and observed highly negative correlation between AFB₁ levels and average weight, weight gain and survival. They also observed marked histological changes in the hepatopancreas of shrimps fed 100 to 2500 ppb AFB₁, 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 AFB₁

The USFDA has regulated the levels of AFB₁ in food commodities to be processed into foods and has established an action guideline of 20 ppb for total aflatoxin. The action level for AFM₁ 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)

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Foods and feeds</th>
<th>Animals</th>
</tr>
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<tbody>
<tr>
<td>12 ppb</td>
<td>Dried fruits and nuts</td>
<td></td>
</tr>
<tr>
<td>5 ppb</td>
<td>Animal feedstuffs</td>
<td>Cattle and sheep</td>
</tr>
<tr>
<td>2 ppb</td>
<td>Animal feed stuffs</td>
<td>Poultry and swine</td>
</tr>
<tr>
<td>1 ppb</td>
<td>Animal feed stuffs</td>
<td>Piglets and chicks</td>
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Table 2.2. FDA Guidelines on maximum levels of aflatoxin in feedstuff (FAO, 2002)

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Animals</th>
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<tr>
<td>20 ppb</td>
<td>Dairy, immature pigs, poultry, animal feeds, fish and shrimp feeds</td>
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<tr>
<td>100 ppb</td>
<td>Breeding animals</td>
</tr>
<tr>
<td>200 ppb</td>
<td>Finishing swine</td>
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<tr>
<td>300 ppb</td>
<td>Beef cattle</td>
</tr>
</tbody>
</table>

2.1.8. Acute toxicity of AFB₁ to various animals

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

Table 2.3. Toxicity levels of AFB₁ to various animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Animals</th>
<th>Oral LD₅₀ levels (mg/kg)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammals</td>
<td>Rats</td>
<td>7.2-17.9</td>
<td>Edds (1973)</td>
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<td></td>
<td>Mouse</td>
<td>9.0</td>
<td>Edds (1973)</td>
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<tr>
<td></td>
<td>Rabbit</td>
<td>0.3</td>
<td>Patterson (1973)</td>
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<td></td>
<td>Guinea pig</td>
<td>1.4-2.0</td>
<td>Butler (1966)</td>
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<td>Hamster</td>
<td>10.2</td>
<td>Wogan (1973)</td>
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<td>Monkeys</td>
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<td>Shank et al., (1972)</td>
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<td></td>
<td>Baboon</td>
<td>2.0</td>
<td>Peers and Linsell (1976)</td>
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<td>Aves</td>
<td>Ducklings</td>
<td>0.36-0.73</td>
<td>Newberne and Butler (1971)</td>
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<td></td>
<td>Turkey Poult</td>
<td>0.5</td>
<td>Butler (1964)</td>
</tr>
<tr>
<td></td>
<td>Rats</td>
<td>7.2 -8.0</td>
<td>Butler (1964)</td>
</tr>
<tr>
<td>Fishes</td>
<td>Rainbow trout</td>
<td>0.5</td>
<td>Wales(1970)</td>
</tr>
<tr>
<td>Crustaceans</td>
<td>Copepod</td>
<td>1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Reiss(1972 a)</td>
</tr>
<tr>
<td></td>
<td>Brine shrimp</td>
<td>14.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Reiss (1972 b)</td>
</tr>
<tr>
<td></td>
<td>Penaeid shrimps</td>
<td>100.5&lt;sup&gt;im&lt;/sup&gt;</td>
<td>Wiseman et al., (1982)</td>
</tr>
<tr>
<td></td>
<td>(P. stylirostris)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>c</sup> = LC<sub>50</sub> at 24 hr (mg/l);  <sup>im</sup> = intramuscular injection
2.2. Effect of other toxins and heavy metals on AFB₁ 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 AFB₁. The presence of dieldrin in the diet with 6 ppb aflatoxin B₁ 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 B₁ 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 B₁ 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 AFB1/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), oestrogenic 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 AFB1, 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 microorganisms; one example is the conversion of Aflatoxin B₁ 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 AFB₁. 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 AFB₁ 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), monoter-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 B1 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 \( \mu \)g /kg of body weight) of AFB1 for 9 days and concluded that ascorbic acid alleviated the negative effects of AFB1 in a dose-dependent manner. Yousef et al. (2003) evaluated the effectiveness of L-ascorbic acid (AA) in alleviating the toxicity of aflatoxin B1 in male white rabbits fed 30 picograms AFB1 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 B1 (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 B1 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 AFB1 exposed Indian major carp, *Labeo rohita* (Sahoo and Mukherjee, 2002). Karakilicik et al.
investigated the effects of vitamin C and E on liver enzymes and other biochemical parameters in rabbits experimentally exposed to AFB₁ (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 AFB₁.

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 AFBI (25 μg/kg) in albino rats (Asuzu et al., 1988).

2.3.4. Efficacy of Food additives, beverages, spices and herbs on AFBI 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 (0.5 μg / plate) in Salmonella strains. Dietary administration of turmeric, garlic, curcumin and ellagic acid to rats significantly reduced the occurrence of hepatocellular neoplasm (Soni et al., 1997). Turmeric and asafoetida were also useful in reducing AFBI production in cultures of Aspergillus parasiticus (Soni et al., 1992). Curcumin, present in turmeric reversed AFBI 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 AFBI.

‘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 B1 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 B₁ by multienzyme isolated from mycelium pellets of Armillariella tabescens. Broilers treated with N-acetylcysteine (NAC) (800 mg/kg body weight) plus AFB₁ (3 mg/kg of feed) were shown to be partially protected against negative effects induced by AFB₁ 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.