Cellular and humoral factors involved in defense mechanisms of *Fenneropenaeus indicus* (H. Milne Edwards, 1837)

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By

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Certificate

This is to certify that this thesis entitled "Cellular and humoral factors involved in defense mechanisms of *Fenneropenaeus indicus* (H. Milne Edwards, 1837)" is an authentic record of research work carried out by Nisha. P. C. (Reg. No.2237) under my guidance and supervision in Central Marine Fisheries Research Institute, in partial fulfillment of the requirement for the Ph. D degree in Marine Science of the Cochin University of Science and Technology and no part of this has previously formed the basis for the award of any degree in any University.

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Date: 09-10-2006

Declaration

I hereby declare that the thesis entitled "Cellular and humoral factors involved in defense mechanisms of *Fenneropenaeus indicus* (H. Milne Edwards, 1837)" is an authentic record of research work carried out by me under the guidance and supervision of Dr. K. C. George, Principal Scientist, Physiology, Nutrition and Pathology Division, Central Marine Fisheries Research Institute, in partial fulfillment for the Ph. D degree in Marine Science of the Cochin University of Science and Technology and no part of this has been previously formed the basis for the award of any degree in any University.

(NISHA. P. C)

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Dedicated

to my family and

teachers

Contents

Introduction

1. Introduction

Aquaculture has come a long way from rearing organisms in traditional ways, in extensive enclosures, mainly for domestic purposes. Today, aquaculture has been molded into a highly export oriented industry involving many sectors of the community. In many countries, especially many Asian and South American countries, aquaculture is synonymous with shrimp culture and forms the main source of foreign exchange. In addition, it is advantageous in other ways as it reduces the pressure of over-fishing; creates sustainable fisheries and alternate occupation for fishing communities. The world shrimp production from aquaculture was 8, 55,500 tons 2002-2003. The contribution of India was 1, 15, 320 tons, which in 1990-91, was a meager 35,500 tons. This increase is mainly due to improvement and intensification of culture practices (FIGIS, 2006).

Intensification in aquaculture practices has resulted in problems of disease, which have caused a reduction in production and this has led to huge economic losses in many countries. In Taiwan, there was a 60% reduction in the production of shrimp in 1987-1988. In Ecuador, the shrimp production decreased by 65% in the year 2000 (Gullian *et al.*, 2004). The development of diseases, particularly in shrimp aquaculture, is the result of intensification of production, based on zootechnical progress, which has led to ecological and environmental disturbances, pollution and nutritional imbalances (Kautsky *et al.*, 2000). Such conditions and possible genetic factors, have contributed to the appearance of non-infectious and infectious diseases (Bachère, 2003). Moreover, the practice of transfer of seed and broodstock at the national and international level has amplified the spread of many pathogens.

A number of viral, bacterial and fungal diseases have surfaced in aquaculture and have affected the culture of all the important cultured species of shrimp, *viz. Penaeus monodon*, *Penaeus chinensis*, *Litopenaeus vannamei*, *Penaeus japonicus*, *Litopenaeus stylirostris*, *Penaeus merguensis* and *Fenneropenaeus indicus*. Baculoviruses like *Baculovirus penaei* (BP), 'Monodon baculovirus' (MBV), parvoviruses such as 'hepatopancreatic Parvo-like virus' or

picorna-viruses like 'infectious hypodermal and haematopoietic necrosis virus' (IHHNV) are some of the main viruses seen affecting the culture operations in many countries. White spot syndrome (WSS) is a major disease faced by Asian countries and is caused by a virus under the genus *Whispovirus* coming under the family Nimaviridae. In addition to these viruses, other pathogens that affect the main cultured species of shrimp are pathogenic bacteria that have always been involved in the diseases of crustaceans in hatchery and growout stages. *Vibrio harveyi*, *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are some of the pathogenic species that are prevailing in the culture environment (Crisp and Bland, 1989; Baticados *et al.*, 1990; Lightner, 1992; Song *et al.*, 1993; Song and Lee, 1993; Karunasagar *et al.*, 1994; Robertson, 1970; Costa *et al.*, 1998; Gullian *et al.*, 2004).

Many microorganisms are opportunistic and become pathogenic due to deterioration of the culture environment. The aquatic environment by and large is affected by anthropogenic activities. Consequently, the water used for culture is a broth of many hazardous substances, which reach the culture site as a result of land drainage. The unscientific and indiscriminate use of drugs and chemicals in aquaculture operations in concert with the contaminants reaching the culture waters through land drainage leads to compromise of the health of farmed animals, thus making them highly susceptible to diseases.

In order to ward off diseases many farmers resort to the use of antibacterial agents like antibiotics. Treatment with antibiotics leads to bacterial drug resistance, tissue residues in farmed shrimp, contamination of culture environment and ecological damage. As the culture waters are invariably linked to the bodies of natural waters, application of antibiotics often affects the natural flora and fauna. Shipment laws have been made stringent in the face of rejection by many developed countries, of consignments containing antibiotic residues. All this has led to efforts for preventing disease outbreaks by adopting prophylactic measures including modulation of the immune system of the farmed animals.

The immune system of invertebrates is characterized by the lack of adaptive mechanisms, which involve antibodies. Adaptive immunity renders

specificity and memory against a particular pathogen. But the absence of such a system is compensated by a dynamic non-specific immune system involving cellular and humoral components. Research on pathology in marine invertebrates is usually descriptive, focusing on the pathogen morphology, anatomorphology and epidemology. Research on host-pathogen interactions requires a thorough knowledge on the nature of the host immune system. Only then can disease prevention strategies based on animal health monitoring and manipulation of defense mechanism be established.

Crustaceans, in general are known to inhabit a wide range of environments, which is dynamic, and ever changing. Being poikilotherms, wide variations are seen in their hematological parameters. Thus, reactions of shrimps and other aquatic invertebrates to the ever-altering environment are unpredictable. As a result of this, studies on the defense factors of individual species during various environmental conditions are essential.

The knowledge on the immune system of crustaceans, especially of penaeid shrimps, is insufficient, compared to their land counterparts. Research on the haematological parameters that also form components of the immune system is scarce. Information on the modulations of these parameters in the face of contaminants especially, pesticides and heavy metals are also scanty. Not only this, the knowledge on variations seen in the immune factors during physio-chemical changes in the environment is also necessary. Only then can efficient prophylaxis be applied in culture applications. Periodic sampling and monitoring of immune factors of cultured species will also help in detecting deficiencies in the quality of the culture environment that increases the susceptibility of the animals to diseases.

The Indian white prawn, *Fenerropenaeus indicus* H. Milne Edwards is widely cultured in India and has a prominent place in the internal as well as external markets. It is widely cultivated in the extensive and semi-intensive fields of Kerala, Karnataka, Orissa and West Bengal. This indigenous species is affected by many pathogens (Soni, 1986; Shahul Hameed, 1989) and is also affected by changes in physio-chemical variations in the culture environment.

Histopathological studies have been conducted in *F. indicus* (Shahul Hameed, 1989; Viswanathan and Manniseri, 1995). Studies on the haemolymph components of this species have been performed (Laxmilatha, 1991; Jayasree and Selvam, 2000; Laxmilatha and Laxminarayana, 2004). Studies on immunology in *F. indicus* are scarce and it is believed that the present study will augment the knowledge on their defense mechanisms and modulations in the face of pathogens and environmental changes.

The main objectives of the present study were

1. to characterize the cellular and humoral factors of immune system in *F. indicus*
2. to understand the behaviour of these factors during the infection of *Vibrio parahaemolyticus*
3. to find out the effect of the organophosphate pesticide 'Nuvan' and the heavy metal zinc on the immune factors
4. to understand the modulations in the cellular and humoral factors from exposure to various salinities and
5. to find out the effect of commercial immunostuimulant 'Allways' on the immune factors of *F. indicus*

Review of Literature

2. Review of Literature

2.1. Crustacean immune system

Crustacea, like other arthropods, have an open vascular system and the circulating fluid is termed haemolymph. Elastic but non-muscular arteries leave the heart, which have valves to prevent the flowing back of haemolymph. These arteries penetrate the pericardial membrane and after a variable number of branching processes deliver the haemolymph to irregular spaces or sinuses, scattered throughout the body. An exchange of substances takes place between the haemolymph and the surrounding tissues and organs. The components of the circulatory system also form the immune system of crustaceans (Bauchau, 1981). Being invertebrates, crustaceans lack the adaptive immunity, which is unique to vertebrates. Vertebrates have a specific immune system, which can mount targeted attacks on the invading offenders. This system possesses memory, giving special advantage to the host. It has both cellular and humoral armours represented by T-killer cells and antibodies.

The immune system of invertebrates lack the adaptive mechanisms mediated by antibodies. Their defense mechanism is based on innate components consisting of cellular and humoral factors that interplay to recognize and eliminate foreign microorganisms and pathogens. The particulate part of the haemolymph, the haemocytes, forms the cellular factor of the non-specific immune system. These cellular components mediate cellular responses like phagocytosis, clot formation, encapsulation and nodule formation. The humoral responses of invertebrates are actually special cases of cellular responses, in which "Cellular secretion, fragmentation, or biochemical alteration confers bacteriostatic, lytic, or other properties on body fluid" (Stauber, 1961). Factors concerning the humoral defense system include phenoloxidase, the prophenoloxidase activating system, bactericidins and lectins (Takahashi *et al.*, 1995; Lee and Shiau, 2003).

2.1.1. Haemolymph factors

2.1.1.1. Cellular factors

2.1.1.1.1. Characterization of haemocytes

Carus studied crustacean haemocytes as early as 1824, and ever since, haemocytes are being studied along two main lines of investigation: the first directed towards the characterization of different morphological types of cells while the other has sought to determine their particular role in blood clotting. There has been great interest in haemocytes due to their involvement in wound repair and defense mechanisms as an open circulatory system is always prone to intrusion from the surrounding environment (Bauchau, 1981).

Several attempts to classify crustacean haemocytes were made over the years. But a lack of logical guidelines in the classification schemes and sufficient caution in sampling procedures has led to a state of confusion in haemocyte classification. This has resulted in many terms and names. In the early studies, haemocyte was known as pale amoeboid cell, amoebocyte hyaline, explosive corpuscle, eosinophilic corpuscle, hyaline thigmocyte, thigmotactic amoebocyte and acidophilic granular cell (Halliburton, 1885; Cuenot, 1891; Hardy, 1892; Tait and Gunn, 1918; George and Nichols, 1948). Cuenot (1891) stated that all blood cells in crustacea originate from a single type of stem cell. More recent research supports this view of a developmental sequence, which successively gives rise to three main forms; hyaline, semi or small granular and granular or large granule haemocytes (Bruntz, 1907; Arvy, 1952; Wood and Visentin, 1967).

The American lobster, *Homarus americanus* was found to possess both granulocytes and non-granulocytes. Based on the shape and staining characteristics, haemocytes were classified into three types; cells containing acidophilic granules, called eosinophil, spindle shaped haemocytes called spindular basophil, containing basophilic granules and oval shaped ovoid basophil, containing basophilic (Hearing and Vernick, 1967). Another classification is that of Cornick and Stewart (1978), who recognized four types of cells in *H. americanus*. These were based on the size and refractile nature of granules, ratios of cytoplasm to nucleus, and Geimsa staining characteristics.

Two hyaline types were designated as prohyalocytes and hyalocytes, and two granular types termed eosinophilic and chromophobic granulocytes. There was no significant variation in the percentage of cells among different sexes. However, the percentage of eosinophils and hyalinocytes differed significantly between different populations of lobsters.

Ultrastructural studies of haemocytes of the blue crab, *Callinectes sapidus* revealed three types of cells *viz*; hyaline cells, intermediate cells and granulocytes. The three types of haemocytes showed the same cytological characteristics such as morphology of organelles, appearance of the nucleus and the presence of similar types of inclusion bodies. These features suggest that the cells of blue crab are inter-related. The differences in size and shape of the cells and their inclusion bodies were thought to be the result of transitions that might be anticipated for cells that differentiate towards a common end point (Bodammer, 1978).

Sternshein and Burton (1980) with the help of light and electron microscopy observed the behaviour of haemocytes of the crayfish, *Procambarus* spp., and *Orconectes* spp. during the clotting process and accordingly classified them into three types. The coagulocyte, formed the main cell population containing medium sized granules and abundant rough endoplasmic reticulum. These cells participated in coagulation. The oval shaped granulocyte had large, fairly homogenous, irregularly shaped granules in the cytoplasm. The organelles comprised of moderately developed Golgi complex and moderate amounts of rough endoplasmic reticulum. The amebocyte had an indented or horseshoe shaped nucleus, small granules and vacuoles of various sizes.

Haemocytes of the Dungeness crab (*Cancer magister*) were classified into three groups of cells using two simple criteria: the presence or absence of cytoplasmic granules and staining characteristics of the granules present. The cells were hyalinocytes, which were devoid of granules; intermediate granulocytes, which contained basophilic granules or a mixture of basophilic and acidophilic granules and eosinophilic granulocytes that contained large, acidophilic granules (Mix and Sparks, 1980).

The unstimulated haemocytes of the horseshoe crab, *Limulus polyphemus* has all the organelles of a metabolically active cell (Copeland and Levin, 1985). However, mitotic figures that represent cell divisions are absent. The distinguishing feature of the cells is the presence of granules. The unstimulated haemocytes have a population of large granules (predominant) and small, asymmetric electron dense granules (not always present).

Martin and Graves (1985) observed three types of haemocytes in two species of penaeid shrimps, *Sicyonia ingentis* and *Farfantepenaeus californiensis*. The agranular cells were the smallest and lacked granules. The small granule haemocytes were the most abundant type, and contained variable number of small, black granules in the cytoplasm. The third type of cell was the large granule haemocyte that had nearly 25-40 large granules in the cytoplasm, which were highly refractile under the light microscope and electron dense under the electron microscope. Benjamin and James (1987) have observed three forms of haemocytes, hyaline, semigranular and granular, in *Ligia oceanica* and opine that the haemocytes are different stages, which form a single developmental series. According to them, the hyaline cell is the undifferentiated stage, semigranular cell stage is the developing state and granular cell is the mature stage.

Tsing *et al.* (1989) have conducted ultrastructural studies in detail in the penaeids *P. japonicus* and *P. monodon* and have distinguished three forms of cells. The undifferentiated cells do not show pronounced signs of differentiation and contain, very rarely, little round cytoplasmic granules. The small granule haeamocytes contain numerous cytoplasmic granules of spherical shape. The large granule haemocytes have numerous cytoplasmic granules, which are often of different shapes. In addition to these, *P. monodon* possesses large granule heamocytes that contain large, elongated inclusions with a granular content.

In the horseshoe crab, *Tachypleus tridentatus*, two kinds of hemocytes *viz.* plasmatocytes and granulocytes were revealed from the studies of Jakobsen and Suhr-Jessen (1990).

Hose *et al.* (1992) have observed the production and release of haemocytes from hematopoietic tissue, during moult cycle of the shrimp, *S. ingentis*. They have also described morphological stages in the transition of hematopoietic stem cells into hyaline haemocytes and granulocytes. Three types of cells were observed in the crayfish, *Procambarus clarki* (Lanz *et al.*, 1993), which were termed as hyaline, semi-granular, and granular. The hyaline cells have a high nuclear/cytoplasmic ratio. The semi-granular cells have numerous, small, round eosinophilic granules and granulocytes have large eosinophilic granules. The studies of Gaargioni and Barracco (1998) have revealed that the main circulating cells in *Farfantepenaeus paulensis* are hyaline haemocytes that contained few to no granules. According to them, unlike penaeids, palaemonids have numerous granules in their hyalinocytes, as is seen in *Macrobrachium rosenbergii* and *Macrobrachium acanthurus*. In these species, it is the small granule haemocytes that formed the main circulating haemocyte. Yip and Wong (2002) have differentiated the cells of *Penaeus penicillatus*, *P. monodon*, and *P. japonicus* into hyalinocytes and granulocytes. The most abundant type of cells in the crab *Potamon fluviatilis* are the semi or small granule haemocytes. The other cells are the hyalinocytes and granulocytes (Yavuzcan-Yildiz and Atar, 2002). The three cells in *F. indicus* were identified as agranulocytes, dense granulocytes and semi-dense granulocytes. The presence of a fourth type of cell, the cyanocyte, was also noted in animals with developing ovary (Laxmilatha and Laxminarayana, 2004). In *F. californiensis*, *L. vannamei* and *Litopenaeus stylirostris*, small granule haemocytes are the predominant members, constituting 51% of the total haemocyte population. Hyalinocytes followed at 29% and large granule haemocytes were the least, at 19 % (Vargas-Albores *et al.*, 2005). .

2.1.1.1.2. Cytochemistry of crustacean haemocytes

Cytochemical examination helps in complementing the physiology of cell types by elucidating the chemical contents of the cell. Crustacean haemocytes perform a variety of functions and this is reflected in their structural composition (Bauchau, 1981).

2.1.1.1.2.1. Carbohydrates and lipids

Carbohydrates in haemocytes are present in many forms. They may be present as mucopolysaccharides or linked to proteins forming glycoproteins. They may also be present as energy reserves, in the form of glycogen. Glycogen in the cytoplasm of haemocytes is reported in *Artemia salina* (Lockhead and Lockhead, 1941) *Pachygrapsus marmoratus* (Arvy, 1952) and *Orconectes virilis* (Wood and Visentin, 1967). In many species of crustaceans, carbohydrates may be present in the cytoplasm or they may be confined to granules present in the cytoplasm. Granules are mainly composed of neutral mucopolysaccharides. In some, acid mucopolysaccharides are also found (Dall, 1964). In *C. maenas* carbohydrates are in the form of glycogen, as deposits in the cytoplasm of hyalinocytes or between the granules of granulocytes (Johnston *et al.*, 1973). Non-glycogen granules present in *Eriocheir sinensis* are tentatively identified as chitin (Johnston and Davis, 1972; Bauchau *et al.*, 1975). This, however, has not been confirmed. William and Lutz (1975) noticed that granulocytes of *C. maenas* contained either glycogen containing granules, or non-glycogen polysaccharide containing granules.

Cytochemical analysis of ridge back prawn, *S. ingentis* revealed that the abundant cytoplasmic deposits of agranular cells and some of the small granule haemocytes are composed of glycoproteins. Such cells are also positive for the presence of lipids. Lipids are present in all cell types as energy reserve. They are usually seen as deposits in the cytoplasm. Otherwise they accumulate in the granules of lipoprotein cells (Hose *et al.*, 1987).

2.1.1.1.2.2. Prophenoloxidase, Acid Phosphatase and Peroxidase

The prophenoloxidase system is a complement like enzyme cascade, responsible for the formation of melanin. In lower vertebrates and in invertebrates, this dark pigment is deposited in the presence of microorganisms. Prophenoloxidase, a zymogen, is converted to active phenoloxidase, the terminal enzyme in the system. Quinones are by-products of this system and are capable

of pathogen destruction. Phenoloxidase is also involved in non-self recognition as well as generation of opsonins and release of cell adhesion proteins (Söderhäll, 1981). Phenoloxidase enzyme is mainly present in the large granule haemocytes. Thus, they are the main performers in encapsulation, which ultimately leads to the deposition of melanin. Phenoloxidase is sometimes seen diffused in the granular and electron dense cytosol of large granule haemocytes. Hyaline cells, as they contain neither acid phosphatase nor phenoloxidase, do not take part in phagocytosis or encapsulation. But they are seen to initiate coagulation (Tsing *et al.*, 1989; Hose and Martin, 1989). Sung *et al.* (1998) confirmed the presence of phenoloxidase enzyme in the haemocytes of the shrimp, *P. monodon*. The majority (90%) of the enzyme is located within the shrimp haemocytes and not in the plasma (Perazzolo and Barracco, 1997).

Hose *et al.* (1987) used acid phosphatase together with the enzymes, β-glucoronidase and non-specific esterase to demonstrate the presence of lysosomes in *S. ingentis*. In this species, acid phosphatase is mainly present in the small granule haemocytes and in some of the large granule haemocytes. Agranular cells or undifferentiated cells of the prawn are negative for acid phosphatase and other enzyme activities. Sub groups of small granule haemocytes that contain glycoprotein deposits also lack acid phosphatase activity.

Tsing *et al.* (1989) observed that in *P. monodon*, the small granulocytes demonstrate acid phosphatase activity. The activity was less in large granule haemocytes. The authors opined that small-granule haemocytes, by virtue of their acid phosphatase, and other lytic enzyme presence, are the main players in phagocytosis. These cells also participate in encapsulation to a less extent. In the crayfish, *P. clarki*, all the three types of haemocytess, hyaline, semigranular and granular cells contain the enzyme acid phosphatase. But phenoloxidase is located only in the granular and semigranular cells (Lanz *et al.*, 1993).

Song and Hsieh (1994) was the first to describe oxidative metabolism in the shrimp *P. monodon*. The reactive oxygen intermediates were investigated and a myleoperoxidase-enzyme like activity was detected. Gargioni and Barracco

(1998) observed that the granulocytes of *M. rosenbergii*, *M. acanthurus*, and *F. paulensis* contain numerous vesicles positive for acid phosphatase. The cytosol of small-granule haemocytes and large-granule haemocytes were rich in carbohydrates. Sung *et al.* (2000) have conducted *in vitro* phagocytic studies in *M. rosenbergii*, during which acid phosphatase was observed in all the cell types. Highest activity was seen in the hyaline cells, followed by semigranulocytes and then granulocytes.

2.1.1.1.3. Total and differential haemocyte count

The total haemocyte count, *viz.* the total number of cells present in milliliter of haemolymph, is an indicator of the number of cells available for defense reactions. The cellular profile or the differential haemocyte count is a picture of the individual types of cells available to the animal (Noga, 2000). Total and differential haemocyte counts provide a useful way of assessing the physiological state of the animal. Unfortunately, for most crustaceans, wide range of values was available and differences in the classification schemes used have prevented the comparison of haemocyte profiles among different crustaceans. The literature on cell numerations in crustaceans is scarce. Work on the effect of various biological and environmental parameters on cell counts is also limited.

Hardy (1892) gave the first account on total cell count in crustaceans (*Astacus astacus*). The total haemocyte counts of 26 species of marine invertebrates show considerable interspecies and intra-species variation (Yeager and Tauber, 1935). The studies of Stewart *et al.* (1967) have revealed that various parameters such as sex, diet, length at captivity and stage in the moult cycle affect the haemocyte count.

Smith and Söderhäll (1983) determined the total haemocyte count of *A. astacus* as 4.1×10^5 cells/ml and of *C. maenas* as 26.5×10^6 cells/ml and observed that the total haemocyte count responds to saline and glucan injections. The total haemocyte count of *S. ingentis* and *F. californiensis* is 13,737 and 10,660 cell/ml, respectively (Martin and Graves, 1985). Benjamin and James (1987) have

determined the total haemocyte count of *L. oceanica* as 1×10^6 cells/ml. The total haemocyte count of *C. sapidus* is reported to be 5.8×10^7 cells/ml (La Peyre and Chu, 1990).

In penaeid shrimps, Chang *et al.* (1999) opined that the total haemocyte count ranged from $20 - 40 \times 10^6$ cells/ml of haemolymph. The total haemocyte count in *P. monodon* is $14 \pm 6\times10^6$ cells/ml (Rengpipat *et al.* (2000) and that in *F. californiensis*, *L. vannamei* and *L. stylirostris* varies between 4 and 14×10^6 cells /ml of haemolymph (Vargas-Albores *et al.*, 2005). The count was low in *F. californiensis* as compared to *L. stylirostris* and *L. vannamei.*

Hearing and Vernick (1967) observed that, of the three cell types in the lobster *H. americanus*, eosinophil forms 20% of the total number. The other two cells containing basophilic granules, the spindular basophil forms 60%, and the ovoid basophil forms 20%. But in the same species, Cornick and Stewart (1978), recorded two hyaline types and two granular types. They were designated as prohyalocytes (1.8%) and hyalocytes (64.2%), and two granular types termed eosinophilic (12.2%) and chromophobic granulocytes (21.9%).

In the crayfish, *Procambarus* spp., and *Orconectes* spp., Sternshein and Burton (1980) found that the coagulocyte, with medium sized granules comprised 65% of the population. The oval shaped granulocyte with large irregular shaped granules constituted 31%. The amebocyte had small granules and formed only 4% of the population. In the Dungeness crab (*C. magister*), the intermediate granulocytes with small basophilic granules constitute 65.97%. This is followed by eosinophilic granulocytes forming 17.76%. The percentage of hyalinocytes is almost similar to the eosinophilic granulocyte and constitutes 16.25% of the total number of haemocytes (Mix and Sparks, 1980).

In the penaeid shrimps, *S. ingentis* and *F. californiensis*, agranular cells constitute 5-10% of the cell population. The small granule haemocytes are the most abundant type forming 75% and the large granule haemocytes comprise 10-20% of the total cell population (Martin and Graves, 1985). In *L. oceanica*, the hyaline to semigranular and granular haemocytes exist in the ratio 8:3:9 (Benjamin and James, 1987). The most abundant type of cell in the crab *P.*

fluviatilis is the semi or small granule haemocyte (54.25%). The hyalinocytes constitute 15% and granulocytes 30.75% (Yavuzcan-Yildiz and Atar, 2002). In *F. californiensis*, *L. vannamei* and *L. stylirostris*, small-granule haemocytes is the predominant type forming 51% followed by hyalinocytes (29%). Large-granule haemocytes are the least (19 %) (Vargas-Albores *et al.*, 2005).

2.1.1.1.4. Phagocytosis

Haemocytes play a number of physiological roles in the crustacean body, from transporting nutrients to hardening the exoskeleton. Microbes gain access to animals when they are weak, particularly, during poor environmental conditions. The haemocytes are the major defenders of the body, as they respond directly as well as indirectly to these microbial invasions. Phagocytosis is a defense mechanism seen throughout the animal kingdom. This direct involvement of haemocytes in warding off intrusion forms the main defense tactic in crustaceans. Encapsulation and nodule formation are alternate ways of defense when the pathogens are larger, or are greater in number, for a single cell to phagocytose them.

The phagocytic capacity has been studied in various crustaceans. Fontaine and Lightner (1974) who studied phagocytosis in *P. setiferus* have opined that the clearance mechanism of foreign materials in the shrimp is very slow. Phagocytosis is accomplished by haemocytes circulating in the haemolymph and by fixed phagocytes in gills, heart, loose connective tissue, and blood sinusoids in the abdomen. Phagocytosed particles are eliminated through the epithelium of the gills, gut, hepatopancreas and extremities of periopods and pleopods. Necrotic haemocytes with phagocytosed material are encapsulated in the periopods. In *P. setiferus*, the encapsulated material remained in the shrimp body up to 33 days before it was eliminated.

The phagocytic cells may be distinguished from others by having abundant endoplasmic reticulum, large droplets and numerous digestive vacuoles. The amoebocytes of spirochete-infected *A. salina* showed such features. Spirochetes were seen in the vacuoles of these cells. In some, they were

morphologically degenerated (Tyson, 1975). Smith and Ratcliffe (1978) observed that the phagocytic cells in *C. maenas* are characterized by variable numbers of small intracellular granules. The phagocytic activity of these cells, studied *in vitro*, using Gram-positive and Gram-negative bacteria and erythrocytes showed that the rate of phagocytosis of different materials by the phagocytic cells varies. It is seen that the rate of phagocytosis of Gram-positive bacteria by the phagocytic cells is low and is not enhanced by serum incubation. In contrast, the Gram-negative bacteria are phagocytosed at a higher rate but serum treatment only depresses phagocytosis.

In certain crustaceans, phagocytosis is restricted to granulocytes. Benjamin and James (1987) are of opinion that in *L. oceanica*, granulocytes alone are capable of phagocytosis. The authors suggest that the various haemocytes of this crustacean is part of a developmental series. Here the hyaline cells are the undifferentiated cell stage. The semigranular cell is the developing stage, and the granulocyte is the mature stage of the developing series of haemocytes. All the cells have pseudopodia and are capable of amoeboid movement. The authors opine that phagocytosis is restricted to granulocytes. The presence of enzymes in granule containing cells may be responsible for this. Tsing *et al.* (1989) observed that the small granule haemocytes contained vesicles abundant in the enzyme phospahtase. The authors opine that these cells play a major role in phagocytosis and endocytosis.

Hose and Martin (1989) state that phagocytosing cells in the ridge back prawn *S. ingentis* increases with time. The percentage of phagocytic small granule haemocytes increases with time. An increase in the phagocytic cells is also seen on opsonization of substrate cells (bacteria). Sagrista and Durfort (1990) are of the opinion that the cells occurring as nodules in the haemal spaces of the hepatopancreas of the decapod, *Palaemonetes zariquieyi*, are phagocytic cells and resemble small granule haemocytes. These cells contain vacuoles that are thought to be secondary lysosomes and act as barriers to pathogens entering through hepatopancreas. Acid phospatase activity is evident in the Golgi complex, primary lysosomes and heterophagic vacuoles of these cells.

The presence of phenoloxidase enzyme plays a prominent role in phagocytic activity of cells. La Peyre and Chu (1990) observed that adhesion and phagocytosis of horse red blood cells by crab haemocytes were low in the haemocytes of *C. sapidus*. The authors conclude that depletion of prophenoloxidase containing granules from small and large granule haemocytes due to external stimulus or disturbances may be the reason for the low phagocytic potential of the cell.

Opsonins are molecules that attach to receptor molecules present on the cell membrane of phagocytic cells. Opsonized phagocytes have a better chance of adhering to substrate molecules. Bayne (1990) has given a detailed description of the process of phagocytosis in invertebrates and has compared it with that of vertebrates. The author has reviewed the studies on opsonins in molluscs and crustaceans and suggested the presence of a variety of opsonins and cell membrane receptors in invertebrates. It is suggested that haemocyte activity can be enhanced by activation with vaccines, due to the presence of phagocyte receptors for plasma opsonins.

Vaccination, however, does not alter the levels of humoral components. Kondo *et al.* (1992) in their studies on opsonization in *P. japonicus* found that all types of cells, *viz.* agranular and granular cells were capable of phagocytosis. Ekapanithanpong *et al.* (1999) too observed that in *P. monodon* and *P. japonicus*, the hyaline and granular cells are capable of phagocytosis of glutaraldehyde-fixed sheep red blood cells. Their studies revealed that serum opsonization increased the phagocytic capacity of the cells and they suggested that opsonins in serum play an important role in phagocytosis.

The granulocytes of *P. japonicus* showed a mean phagocytic capacity of 71.7 ± 4.9 % (Itami *et al.*, 1998). Vargas-Albores *et al.* (2005) separated the haemocytes of *F. californiensis*, *L.stylirostris* and *L. vannamei* by Percoll gradient centrifugation, and studied the prophenoloxidase activity and discrimination ability of each cell type. Prophenoloxidase activity was seen in the granule-containing cells, mainly the large-granule haemocytes, which showed more discriminating and phagocytic ability.

2.1.1.1.4.1. Superoxide formation

Reactive oxygen species (ROS) or reactive oxygen intermediates (ROIs) formed during oxygen metabolism are toxic compounds to the cells. They are superoxide ion radicle (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and the most reactive species, the hydroxyl radicle (^-OH). In defense reactions, the ROS are directed against intruders, resulting in their destruction. During phagocytosis, phagosomes with internalized particles or microorganisms are formed in the cell. The elimination of phagocytosed particles involves the release of degradative enzymes into the phagosome and generation of ROS. The production of ROS is called the respiratory or oxidative burst (Babior, 1980). After the haemocytes bind to the foreign material, NADP oxidase, the membrane-bound enzyme complex, reduces molecular oxygen to superoxide anion, which is the first ROS generated in the respiratory burst. Superoxide anion is formed in reactions where molecular oxygen is present. This is readily scavenged by the enzyme superoxide dismutase (SOD) to form hydrogen peroxide and oxygen.

NBT reduction assay indicates the presence of superoxide anion. NBT reduction is not totally specific for superoxide and its specificity is controlled by superoxide dismutase. The result of NBT reduction assay is generally reported as SOD-inhibitable activity. In the presence of O_2^-, pale yellow NBT is converted to a dark blue, water insoluble formazan product that can easily be visualized in the cytoplasm of phagocytes.

Literature on the respiratory burst in invertebrates mainly concerns molluscs (Dikeboom *et al.*, 1985; Bachère *et al.*, 1991; Pipe, 1992). Bell and Smith (1993) first demonstrated the production of ROIs in crustacean haemocytes. In *C. maenas* hyaline cells produce superoxide anion. Song and Hsieh (1994) described for the first time the oxidative metabolism in penaeids, in *P. monodon*. Bachère *et al.* (1995) demonstrated the existence of respiratory burst in *P. japonicus*.

Muñoz *et al.* (2000) observed a failure to produce respiratory burst by *L. vannamei* on exposure to *V. alginolyticus*. The unstimulated animals expressed a decrease in oxygen radical production. The authors opined that earlier stimulation by a pathogen would have produced oxygen burst in the unstimulated animals, which with time, would have been nullified by the various anti-oxidative enzymes, thus reducing in the respiratory burst. Yeh *et al.* (2004) recorded increased superoxide production in *L. vannamei* on exposure to increased concentration of copper sulphate and suggested that this increased superoxide production at higher concentrations of copper is cytotoxic to the animal.

2.1.1.2. Humoral factors

Humoral factors that originate or reside in the haemocytes are released into the fluid part of haemolymph by cell lysis or fragmentation. These are responsible for the clotting of haemolymph and have the ability to inhibit microbial growth. Haemolymph of many crustaceans including different species of shrimp, crabs, lobsters, isopods, and stomatopods are known to show broad-spectrum anti-bacterial activity (Khoo *et al.*, 1999). "Endo-biotics" or "host-induced antibiotics" are natural antimicrobial agents produced by the organism that can inhibit or kill bacteria and other pathogens (Noga, 2000). Other humoral factors found in the haemolymph are sugar-binding proteins called lectins that agglutinate foreign particles and aids easy phagocytosis.

2.1.1.2.1. Total protein concentration

Serum is the fluid part of the haemolymph excluding the haemocytes and the clotting protein, coagulogen. Total serum protein represents the dissolved protein content of the serum. This includes the copper containing protein, haemocyanin and various enzymes that confer lytic and restrictive properties to the serum.

The fluctuations in the total protein are expected in a natural population. Three to four fold differences were seen in the serum protein concentrations of

Maia squinado (Drach and Teissier, 1939). Webb (1940) observed a wide range of protein concentration in *C. maenas*. Vast ranges in total serum protein concentrations are seen in many crustaceans (Leone, 1953) including decapoda (Florklin, 1960). Balazs *et al.* (1973) studied the base line serum values of freshly captured Malaysian prawns (*M. rosenbergii*) and pink shrimp (*P. marginatus*). They observed sex differences in serum constituent levels within species. Variations within individuals as well as during the day were observed in *S. serrata* (Subhashini and Ravindranath, 1980).

Smith and Dall (1982) have studied the blood protein, blood volume and extracellular space in *P. esculentus* and *P. plebejus*. The authors suggested that the changes in protein concentration are due to the changes in blood volume. Changes in blood volume take place due to metabolization of muscle tissue as organic reserves. Cameron and Mangum (1983) have reported that haemocyanin, the oxygen transport protein, also functions as an energy reservoir, osmoregulator and buffer. Haemocyanin forms 70% of the total protein, and thus haemocyanin and total protein are well correlated. Gondko *et al.* (1984) conducted studies on protein and ionic content of the haemolymph in three species of Oniscoidea. Laximilatha (1991) characterized protein concentration and protein fractions in the haemolymph of *F. indicus* according to size, sex and moulting stage of animals and found variations in the protein content with size and moult stages. Jayasree and Selvam (2000) studied the effects of mud bank formation on various haemolymph parameters of *F. indicus*. They stated that protein content of the shrimp from mud bank areas record greater protein concentration than those from non-mudbank areas. Acharya *et al.* (2004) have conducted base line studies on the humoral defense factors of the Indian River prawn, *M. malcolmsonii*.

2.1.1.2.2. Electrophoretic studies in crustaceans

The electrophoresis of blood in fifteen species of marine and terrestrial amphipods and isopods has revealed the presence of haemocyanin and other proteins (Wieser, 1965). Stewart *et al.* (1966) conducted electrophoretic and

sedimentation analysis on the plasma and serum of the lobster, *H. americanus*. Purified haemocyanin and fibrinogen prepared from plasma were also subjected to electrophoresis. Cuzon and Ceccaldi (1972) detected the presence of 17 fractions in *Penaeus kerathures*. Alikhan and Lysenko (1975) separated the haemolymph proteins of the isopod, *Porcellio laevis* by SDS-PAGE into 17 fractions. Of these, 3 were haemocyanins, one lipoprotein, one glycoprotein and one esterase. Starvation for 15 days showed a decrease in haemocyanin by 10% and disappearance of the glycoprotein.

Gilles (1977) investigated the osmotic stress on protein concentrations and blood patterns of different crustaceans. Electrophoretic studies to discern the zinc-binding protein in *C. maenas* revealed that the serum contained 7 groups of protein fractions (Martin *et al.*, 1977). SDS-PAGE of the haemolymph showed 3 protein bands in *P. laevis*, 5 in *A. vulgare* 8 in *Orconectes propiquus* (Alikhan and Aktar, 1980) and 16 major fractions in *Squilla mantis* (Ferrero *et al.*, 1983). Prathibha (1984) conducted electrophoretic studies in *P. monodon* and observed 17 protein fractions, which increased with size, but showed no marked difference between males and females.

Invertebrate cells produce anti-microbial peptides. These peptides act in different ways. Some interfere with the permeability of microbial membrane and/or produce pores in the cell membrane (Shai, 1998). Broad-spectrum antibacterial activity is present in the haemolymph of most crustaceans. In *C. pagurus*, and *C. maenas*, squat lobster *Galathea strigosa*, Norway lobster *Nephrops norvegicus* and in the common shrimp *C. crangon* haemolymph associated antibacterial factors are synthesized and are seen to reside in the haemocytes. In *C. maenas* and *A. leptodactylus*, these factors are present in the granule-containing haemocytes. Many of these are small polypeptides of various molecular weights (Chisholm and Smith, 1992; 1995; Schnapp *et al.*, 1996; Khoo *et al.*, 1999).

2.1.1.2.3. Agglutinins

Invertebrates neither possess antibodies nor the complement system. These facts support the hypothesis that recognition of foreignness by invertebrate phagocytes is mainly non-specific and based on antagonistic surface charges or hydrophobic features of the foreign particle's surface. The binding of non-self materials is based on the presence of carbohydrate-specific recognition molecules. These molecules are called lectins, or agglutinins, and occur in the serum of invertebrates.

Blood of the hermit crab *Eupargus prideauxii* contained natural agglutinins that agglutinate rabbit and sheep red blood cells without stimulus or injection of the antigens (Cantacuzène, 1913). The blood of *E. bernardus*, common lobster, *Homarus vulgaris* and spider crab, *M. squinado* has the ability to agglutinate mammalian red blood cells (Cantacuzène, 1921). Solan *et al.* (1975) observed that agglutinins bind specifically and reversibly to carbohydrate determinants of soluble or membrane-integrated glycoproteins or glycolipids, or to the bacterial glycocalyx, which is defined as a polysaccharide containing component outside the cell wall. The authors found that the crayfish, *P. clarkii* could differentiate between bovine serum albumin, human gamma globulin, keyhole limpet hemocyanin and egg white lysozyme.

Most of the agglutinins are of high molecular weight and are dependent on metal ions for their proper function. They are inhibited by many sugars. The lectin found in *P. monodon* is specific to N-acetylneuraminic acid. The purified lectin, termed monodin, is scialic acid specific and is a glycoprotein. It has a molecular weight of 420 kDa and a sub-unit of molecular weight, 27 kDa. The lectin is dependent on calcium ions for its proper function (Ratanapo and Chulavatnatol, 1990).

Haemolymph of the red rock lobster, *Jasus novachollandidae*, agglutinates human ABO erythrocytes and marine bacterium *Pseudomonas aeruginosa*. The haemagglutinating activity is inconsistent and varies from a titer of 4 to 1024. It is heat labile and its activity depends on the presence of Ca^{2+}. It has a molecular weight of 400 kDa and dissociates into subunits of different

molecular sizes (85, 81, and 63 kDa). Porcine stomach mucine (PSM), asialo-PSM, fetuin and several simple sugars are effective inhibitors of agglutinin. Another species of rock lobster, *Jasus edwardsii* also has lectins similar to *J. novachollandidae*. But the green rock lobster, *Jasus verreauxi* has humoral lectins that are sialic-acid specific, and does not depend on Ca^{2+} presence for the proper functioning (Imai *et al.*, 1994).

The haemolymph of *P. japonicus* contains multiple lectins, which are a mixture of agglutinins. The agglutinins agglutinate horse, sheep and human erythrocytes, but not the bacterium, *V. anguillarum*. The activity is dependent on the presence of calcium ions. It is also temperature dependent, giving higher titers at lower temperatures. Glycoproteins and simple sugars are inhibitors of this agglutinin. The two lectins of *P. japonicus* are of the molecular weights 370 and 240 kDa. The first fraction has subunits of 84 kDa and the second has a weight of 34 kDa (Muramoto *et al.*, 1995).

Peng *et al.* (2001) have observed haemagglutinating activity in the haemolymph and haemocytes of *P. chinensis*. This agglutinin is able to agglutinate human A, B and O-type blood cells, rabbit erythrocytes and chicken erythrocytes. The injection with paramyxovirus-like virus is found to change the haemagglutinating activity of the haemolymph.

Maheswari *et al.* (2002) have characterized a natural agglutinin from *F. indicus*. Studies showed that this agglutinin was able to recognize acetyl groups in carbohydrates, amino acids and chemicals and its activity was susceptible to inhibition by lipopolysaccharides from diverse Gram-negative bacteria. It also agglutinated several bacterial species from infected shrimps. Haemagglutinin of *M. malcolmsonii* has been found to agglutinate a wide range of vertebrate erythrocytes, and is stable over a wide range of pH and temperatures (Acharya *et al.*, 2004).

2.1.1.2.4. Enzyme profile

2.1.1.2.4.1. Phenoloxidase enzyme

Melanization is a well known response to infection and cuticular wounding. It is induced by a complex enzymatic cascade, which involves the inactive form of the enzyme, prophenoloxidase and its active form phenoloxidase. According to Söderhäll and Smith (1986), the most important step in overcoming systemic infection in arthropods is the recognition of and the response to non-self entities by the haemocytes. The prophenoloxidising system, which is responsible for converting prophenoloxidase into active phenoloxidase during the early stages of melanization, plays a major role in mediating non-self recognition and host defense in arthropods. The prophenoloxidase (proPO) system has been compared to the mammalian complement system. They seem analogous to each other. The obvious difference is that, the complement system is found in the mammalian plasma, where as proPO system is contained in the haemocytes. The presence of microbial entities like β-1, 3 glucans or bacterial lipopolysaccharides (LPS) stimulates the membrane receptor cells of haemocytes. This recognition of non-self by haemocytes leads to the release of proPO to plasma. Inactive serine protease enzyme is also converted to active serine protease by the presence of glucans and LPS. The active serine protease converts prophenoloxidase into phenoloxidase. Phenoloxidase oxidizes phenol to quinones that polymerize non-enzymatically to melanin (Perazzolo and Barracco, 1997). Melanin and quinones are antimicrobial. Phenoloxidase may also act as possible opsonins that complement phagocytosis.

Phenoloxidase activity was observed in the large granule haemocytes of *S. ingentis* (Hose *et al.*, 1987) and *P. monodon* (Tsing *et al.*, 1989). However, in *S. ingentis,* the small granule haemocytes also possess the phenoloxidase enzyme and both these granulocytes take part in encapsulation reactions (Hose and Martin,1989). The enzyme phenoloxidase from the haemolymph of *F. indicus,* when partially purified, showed high affinity for the biphenolic substrate, adrenalin. This was followed by dopamine, catechol, and dopa. The optimum temperature for enzyme activity was 50 °C, but loses 60% of its activity when

heated to 70°C for 10 minutes. Adrenalin, at a concentration of 15 mM, EDTA, mercaptoethanol and cupric salts, inhibits enzyme activity. Magnesium and calcium salts stimulate enzyme function (Anilkumar and Sridhar, 1993).

Phenoloxidase was extracted and partially purified from *P. japonicus*. The enzyme was found to be stable between pH 5.0 and 8.0, and was most stable at pH 7.0. It was heat stable at 50°C, and was inactivated at a temperature above 50°C. The enzyme had different substrate specificities for different phenolic compounds, showing a maximum activity with triphenol (pyrogallol), then with diphenols (pyrocatechol and L-DOPA) and monophenol (L-tyrosine). The presence of 15 mmol/L ascorbic acid and L-cysteine inhibited 89.6% and 86% of the enzyme activity, respectively (Zhao *et al.*, 1997).

Experiments conducted on haemocyte lysate supernatant (HLS) of *P. paulensis*, have shown that majority of phenoloxidase enzyme is seen in the hemocytes. Serum is also found to contain considerable amounts of the enzyme. The HLS contains a degranulating and cells-adhesion factor. Isolated monolayers of cells incubated in HLS results in the degranulation of a majority of small granule and large granule haemocytes. Both cell types adhere strongly to glass coverslips coated with HLS. Lipopolysachharides, β-1, 3glucan and the proteinase trypsin are able to enhance the phenoloxidase activity of the haemocytes (Perazzolo and Barracco, 1997).

The phenol oxidase activity of *P. monodon* and *M. rosenbergii* is the highest at 37°C and varies according to the concentrations of elicitors. Lipopolysaccharide at 0.5 mg/ml, β glucan and Zymosan at 1 mg/ml and *Vibrio* cells at 10^6 cells/ml elicits phenoloxidase activity. Trypsin is found to increase the enzyme activity in the giant freshwater prawn. Protease inhibitors decrease the phenoloxidase activity in prawn, but in tiger shrimp, neither trypsin nor the inhibitors have any effect. Addition of magnesium and calcium ions increases the enzyme activity, and metal ions at 20 mM give the maximum enzyme activity. However, EDTA hinders the enzyme activity (Sung *et al.*, 1998).

In the cray fish, *P. clarkii*, phenoloxidase was isolated from haemocytes. It was activated by trypsin and zymosan and is calcium dependent. The enzyme

activity in oxidizing L-DOPA is the highest when calcium concentration is 5 mM. When the enzyme is treated with bacterial lipopolysacharides (LPS), there is a lag time of 25-30 minutes, before the reaction starts. Some derivatives of LPS are not able to activate the system. Prophenoloxidase is activated to phenoloxidase by a sereine protease. It is believed that an endogenous sereine protease mediates the recognition of LPS molecule in the activation of prophenoloxidase (Cardens and Dankert, 1997).

Prophenoloxidase from haemocytes of the kuruma prawn, *P. japonicus* has been purified and characterized. It has a molecular weight of 330 kDa with subunits that weigh 78 and 72 kDa. The optimum pH of phenol oxidase activity in this species is 9 and the optimum temperature, 37^0C. Its activity depends on the concentrations of Mg^{2+} and Ca^{2+} and the highest activity is obtained at a concentration of 50 mM for both ions. It can oxidize mono phenols and o-diphenol derivatives, but it is unable to oxidize the p-diphenol derivatives (Adachi *et al.*, 1999).

Gollas *et al.* (1999) have characterized prophenoloxidase from the haemocytes of the brown shrimp, *F. californiensis*. It is a monomeric protein of molecular weight 114 kDa and is hydrolyzed by proteinases to phenoloxidase of 107 kDa. The isoelectric point of both the proteins is 7.35. It has an optimum pH of 7.3 during its reaction with L-DOPA as substrate. Sodium azide, thiourea, and EDTA poorly inhibit the enzyme activity, whereas, it is strongly inhibited by diethyl thiocarbamine. According to substrate affinity and inhibition characteristics, the enzyme is classified as a tyrosine-like phenoloxidase. In this case, purified prophenoloxidase is not activated by bacterial lipopolysacharides or beta-glucans.

Presence of microbes is known to activate phenoloxidase activity in crustaceans (Huang *et al.*, 2000). Significant difference in the phenoloxidase activity was observed between clinically normal and sick *P. ornatus* (Norton *et al.*, 2000).

2.1.1.2.4.2. Acid and alkaline phosphatase enzymes

Acid and alkaline phosphatase enzymes are lysosomal enzymes of low substrate specificity. These enzymes hydrolyze a large variety of organic phosphate esters to form alcohol and phosphate ions. Their probable function is the transfer of phosphate group from a donor substrate to an acceptor compound containing a hydroxyl group. Thus, these are involved in several processes such as dephosphorylation, degradation of mucopolysaccharides, hydrolysis of acylglycerol and hydrolization of peptides bearing free amino acid groups. Acid phosphatase is activated by acidic pH while alkaline phosphatase requires alkaline pH.

Lysosomal enzymes like acid phosphatase, β-glucanase, non-specific esterase and peroxidase have been identified in the haemocytes of many crustacean and these may be released into the haemolymph on stimulation (Tsing *et al.*, 1989; Hose *et al.*, 1990).

Balazs *et al.* (1973) compared the base line values of serum constituents of *M. rosenbergii* and *P. marginatus* with those of animals kept in the laboratory. The serum alkaline phosphatase of laboratory held animals was more than that of freshly caught animals. Cheng and Rodrick (1975) have quantified the acid phosphatase activity in serum and haemocytes of the oyster *Crassostrea virginica.* Omkar and Shukla (1985) have studied alkaline phosphatase and aetylcholine esterase activity and their inhibition due to dichlorvos toxicity.

Cheng and Downs (1988) separated different haemocytes of the oyster, *C. virginica* and determined the levels of acid phosphatase and lysozyme in the various haemocyte subpopulations.

Alkaline phosphatase is a common enzyme seen in almost all species of animals. But the biologic relevance is poorly understood as its optimum pH level often exceeds the normal physiologic range. Poelstra *et al.* (1997) hypothesize that negatively charged groups formed during dissociation of acidic groups in protein synthesis, promote activity of the enzyme. Their studies on the effect of endotoxin on alkaline phosphatase activity suggest that phosphorylated endotoxin supplies negatively charged groups that activate the enzyme at a

physiologic pH. *In vivo* experiments show that the endotoxin toxicity is significantly reduced after exposure to alkaline phosphatase preparations. This implies that alkaline phosphatase accomplishes protection against endotoxin of Gram-negative bacteria after an infection with these micro organisms. Chen *et al.* (2000) studied the acid phophatase activity of *P. penicillatus* and the effect of methanol on this enzyme. The tissue phosphatases of *M. rosenbergii* were studied by Ramalingan and Ramarani (2004). Bhavan and Geraldine (2004) have determined the quantitative and qualitative aspects of acid and alkaline phosphatases in *M. malcolmsonni.*

2.2. Immunomodulation

Research in the field of crustacean immunity has revealed that they have the arsenal to defend themselves from disease causing agents. The increasing changes in the environment due to natural and more often man-made, causes compromise of their immune system. The pathogenic effect of various microbial agents is amplified due to alteration of the culture environment. Knowledge on the action of cellular and humoral factors of crustaceans, particularly shrimp, during pathogenic attacks and during the presence of various environmental conditions and contaminants is scanty. Research in the use of haemolymph factors as crustacean health markers is encouraging, in order to study the effects of environmental deterrents and in turn, to control their effect on culture animals.

2.2.1. Effect of micro organisms

2.2.1.1. Cellular factors

With the rapid growth of shrimp industry, the increasing threat posed by pathogenic organisms has been recognized. Twenty viral species have been recognized from diseased shrimp (Lightner and Redman, 1998) and new species are being discovered. A number of bacterial species have been associated with shrimp systemic septicemia and/or mortality. Although studies have been conducted on the etiology and pathogenicity of crustacean pathogens, work on the effect on immune system of target organisms is scanty

Gaffkaemia of lobsters is a disease that stays apart. One of the most studied pathogens of crustaeceans is *Aerococus* (*Gaffkya*) *homari*, which causes "Gaffkaemia" or "shell disease" that results in massive mortalities in the lobster, *H. americanus*. Cornick and Stewart (1968) have studied the effects of bacterium on defense factors of the haemolymph. They found that the serum of lobsters promotes pathogen growth and in infected animals, phagocytic activity of the haemocytes is greatly reduced. Stewart *et al.* (1969) observed that the main effect of infection was a drastic decrease in the number of circulating haemocytes resulting in an increase in haemolymph clotting time. The number of haemocytes decreased to 50% by the fifth day. By the 11^{th} day they reached $1000/mm^3$ as compared to $22,000/mm^3$ at the beginning.

Ratcliffe and Gagen (1976, 1977) observed the reduction in total circulating cells in the wax moth, *Galleria mellonella*, during the clearance of bacteria. The authors state that the haemocytes clump around the foreign particles to form numerous small nodules throughout the body. The granular cells release substances that activate haemocytes to clump together and entrap the bacteria. In *C. maenas,* bacterial injection caused a 90% reduction in total haemocyte count with in thirty minutes (Smith and Ratcliffe, 1980). Although the total count became normal after twenty-four hours, it was significantly less than that of untreated crabs. The refractile cells and the phagocytic cells of the crab showed significant reduction in the initial stages. Bang (1983) observed a transmissible disease detected by low clotting is accompanied by low numbers of circulating haemocytes. The author opines that the reduction in the circulating cell number is due to selective adherence of cells within vascular channels.

Presence of pathogens also causes increase in the haemocyte count of crustaceans. Söderhäll and Cerenius (1992) observed a dramatic increase in the free haemocytes of cray fish after injections with the parasite *Psorospernium hacheli.*

Trypanasoma cruzi promotes the spreading and degranulation of different populations of the haemocytes of *P. leniusculus*. The crayfish haemocytes are able to phagocytose the epimastigote forms of *T. cruzi* (Barracco and Söderhäll,

1996). Hauton *et al.* (1997) conducted experimental inoculations of the Gram-negative *Listonella* (*Vibrio*) *anguillarum* on *C. maenas*. Infection with sub-lethal doses of live bacterium was monitored for a week. A decrease in the circulating haemocytes, which was maintained throughout the week, was seen during the experiment. A significant increase in the ratio of granulocyte to hyalinocyte was also seen in the animals. Concentration of hydrogen peroxide produced during phagocytosis was enhanced after 7 days of inoculation.

In *P. monodon* infected with *V. harveyi*, the immune system was compromised. Total haemocyte count, phenoloxidase activity and the phagocytic activity decreased in *P. monodon* infected with the luminescent bacterium (Supamattaya *et al.*, 2000). Vogan and Rowley (2002) studied the immunologic differences in *C. pagrus* due to shell disease syndrome. The syndrome displays characteristic black-spot lesions in regions of exoskeletal degeneration. There was no correlation between the total haemocyte count and the degree of infection. But the differential haemocyte counts of the infected animals showed some change with the disease. The percentage of basophilic and eosinophilic granulocytes increased in severely affected animals.

2.2.1.2. Humoral factors

The agglutinating activity of the serum of *H. americanus*, when tested against many bacteria including *A. homari*, is ineffective against the pathogen. Stewart *et al.* (1969) observed that the hepatopancreatic glycogen levels and haemolymph non-protein nitrogen concentrations dropped to a minimum in *H. americanus* infected by *A. homari*. There was significant decline in the infected animal's serum protein content. Although there was a slight reduction in the fibrinogen content of the haemolymph, this did not seem to affect the clotting mechanisms of the animal.

Mohandas *et al.* (1985) with the help of scanning microscopy, have studied the release of lysosomal enzymes in the clam, *Mercenaria mercenaria* challenged with bacterium *Bacillus megaterium*. The study reveals that challenged clams show more lysosmal production than control, and intact

lysosomes are released into serum concurrently with phagocytosis. These enzymes cause partial degeneration of bacterium and enhance endocytosis. Thus the normal process of degranulation is enhanced by the stimulation from phagocytosis of bacteria.

Le Moullac *et al.* (1997) carried out experimental infections in *L. stylirostris* during different moult stages. Shrimp was more prone to *Vibrio* AM23 infection during the premoult stage than the intermoult. The authors suggest that this is due to low phenoloxidase activity during the period. In moribund *P. chinensis* challenged with *Vibrio*.spp, the serum phenoloxidase activity increased. In normal animals, challenge with *Vibrio*.spp, caused little change in the serum phenoloxidase activity. But prophenoloxidase and phenoloxidase activity increased greatly in shrimps challenged with a lower density of the bacterial cell suspension (Li *et al.*, 1998).

The effect of the parasite *T. cruzi* on defense factors of *P. lenisculus* was studied by Barracco and Söderhäll (1996). Incubation of the crayfish haemocyte lysate with fixed epimastigote forms of the parasite induces marked activation of prophenoloxidase system, measured as phenoloxidase activity. In *C. maenas* (Hauton *et al.*, 1997), inoculation of sub-lethal doses of live bacterium, *V. anguillarum* decreased the phenoloxidase activity after 24 hours. But after 7 days, the enzyme concentration increased.

Agglutinin titer values are seen to vary during times of natural infection and could be used as possible indicators of health. Norton *et al.* (2000) conducted haemagglutination tests in clinically normal and sick adult tropical rock lobster, *P. ornatus*. Highly significant difference was obtained in the agglutination values.

In *C. pagrus* affected by shell disease syndrome, serum protein levels decreased with severity of disease. Highly significant negative correlations existed in association with total body lesion cover and ventral surface lesion cover. The prophenoloxidase activity in the haemocyte lysate supernatent showed no correlation with the severity of the disease. But degree of melanisation in diseased animals was lesser than healthy ones (Vogan and

Rowley, 2002). The endotoxin of *P. aeruginosa* (MTCC 1988) increased the activity of acid and alkaline phosphatase in the haemolymph of *M. rosenbergii* (Ramalingam and Ramarani, 2004).

2.2.1.3. Histology

Virchow (1858) was the first to carry out cellular investigations and since then histopathological examination of fixed body fluids and tissues formed a significant and powerful tool for carrying out proper diagnosis of diseases in different organisms. Histo and cytopathological examinations have been carried out by Lightner and Redman (1988) on four penaeid shrimps affected by parvo-like virus. The principal lesion is necrosis and atrophy of the hepatopancreas accompanied by large prominent basophilic, PAS-negative, Fuelgen-positive intranuclear inclusion bodies.

Soni (1986) carried out for the first time in India, histopathological investigations of diseased penaeid prawns. The author observes thickening of the cuticle and haemocytic infiltration and encapsulation in the lobes of tumor-like outgrowths on the dorsal side of the carapace of *P. indicus*.

Lightner and Brock (1987) studied the lymphoma-like neoplasm arising from haemopoietic tissue in *P. vannamei* and suggest the role of viral infection in the development of lesions in the animal. Shahul Hammed (1989) studied the pathobiology of juvenile and postlarvae of *P. indicus* affected by *Vibrio* sp.

Tessprateep *et al.* (1990) have described the histological changes in *P. monodon* due to *V. alginolyticus* infection. Haemocytic aggregation with necrotic core, infiltration of free haemocytes and haemocytic encapsulation was seen in gills, antennal gland, lymphoid organ and hepatopancreas. In severely affected shrimps, septicemia and decrease in the number of free haemocytes were also observed.

Costa *et al.* (1998) have conducted histopathological studies on moribund *L. stylirostris*. Histological sections showed numerous pycnotic cells and intracytoplasmic basophilic bodies together with classical vibriosis lesions. The

authors suggest that the mortality episodes of *L. stylirostris* called 'Syndrome 93' resemble 'Yellow head disease' and 'Taura syndrome'.

Giant cells have been located in the heart tissue of *P. monodon* showing gross signs of Yellow head syndrome (Mohan and Shankar, 1999). The giant cells were observed in the heart lumen and myocardial bands. They were spherical with an eccentric, marginated nucleus. Large numbers of intracytoplasmic inclusions were seen in many of the cells. Some appeared to be phagocytosed material in the cytoplasm. The majority of cells had spherical or horseshoe shaped intracytoplasmic vacuoles. The basophilic intracytoplasmic inclusions imply that they are 'Yellow Head Virus' infected. The authors suggest that they are satellite cells lining the cardiac myocardium, which are often phagocytic and accumulate various materials within the cytoplasm.

Electron microscopy has been used to study the effects of *Thelohania duorara*, which causes cotton shrimp disease in crustaceans. Squash preparations show denatured myofibrils and a loss of cellular structure. Numerous spores are also seen. Ultra structurally, the microsporidians gradually degenerate the myosin and actin fibres, eventually replacing these with spores. In the early phase of infection, small portions of the muscle are affected. As it progressively spreads to greater areas, the muscle fibres are gradually transformed into crystalline structures that eventually appear as electron-lucent components in the remaining muscle tissue. Mitochondria and sarcoplasmic reticulum are degraded (Ramaswamy *et al.*, 2000).

2.2.2. Environmental pollutants

2.2.2.1. Changes in cellular and humoral factors

2.2.2.1.1. Pesticides

Dichlorvos is a contact and stomach-affecting insecticide and is used as a household and public health insecticide. Dichlorvos, as the active ingredient of Nuvan 500 EC or Nuvan 50 EC, has been applied to control ectoparasite crustacean infections, *Lepiophtherius salmonis*, in finfish culture. It has been used widely in the salmon culture operations in Scandinavia and United

Kingdom. Trichlorofan is another ectoparasiticide in finfish culture, most effective against monogeneans infecting gills of sea bass and sea breams. It rapidly degrades to dichlorvos. Dipterex, dursban, demerin and malathion are organophosphates employed in the control of ectoparasitic crustaceans in freshwater fish and monogenic trematode infections in shrimp hatcheries. These are also applied to eliminate certain parasites and their molluscan and crustacean intermediate hosts.

The principal routes for dichlorvos into waters include industrial effluents and accidental discharges, especially from pesticide manufacturing plants, formulation plants and marketing outlets, disposal of unused insecticide and cleaning of application and mixing equipment. Dichlorvos may also indirectly enter the aquatic environment *via* spray drift during application and in land run-off. Effects of organophosphates on non-target aquatic organisms, particularly crustaceans are a major concern. Organophosphates are neurotoxic. It is known to inhibit the activity of the neurotransmitter acetylcholine esterase. Use of organophosphates in agriculture and subsequent run-off of residues into nearby water bodies has been linked to acute effects on crustaceans (GESAMP, 1997).

Omkar and Shukla (1984) have experimentally exposed the fresh water prawn, *M. lamerei* to lethal doses of dichlorvos. The chemical caused a decrease in the acetylcholine esterase and alkaline phosophatase activities, but enhanced acid phosphatase activity.

Egidius and Møster (1987) carried out survival tests on crabs, lobsters and blue mussels with the pesticides, Nuvan and Neguvaon. Nuvan and Neguvon contain Dichlorvos. The animals were treated with 10, 1, 0.5, and 0.1 ppm neguvon and also with nuvan at the rate of 1, 0.1, and 0.01 ppm, respectively. *Homarus gammarus* was the most susceptible species, which died within 24 hours at 0.5 ppm Neguvon and higher and 0.1 ppm nuvan and higher. Blue mussels and the crab, *C. maenas*, seemed to be sturdier than the lobster. There was significant elevation in the serum proteins of *S. serrata* exposed to 5 and 100 mg/l of malathion for three weeks. Activities of the enzymes, aspartate,

aminotransferase and alanine aminotranserfase also increased (Kulkarni and Kulkarni, 1989).

Wichitking *et al.* (1990) have studied the 72 hourLC $_{50}$ of some insecticides and herbicides to the postlarvae of *P. monodon*. The 72 hourLC $_{50}$ was 8 ppm for Daitaphos (Dichlorvos).

Reddy and Rao (1990) have observed the effects of organochlorine pesticides on acid and alkaline phosphatase activity in the shrimp, *M. monoceros*. *In vitro* addition of aldrin and lindane, at different concentrations, inhibits the activity of these two enzymes in various tissues of shrimp. The inhibition causes disturbances in normal protein synthesis.

Geraldine *et al.* (1999) have noted alterations in various parameters in different tissues of the fresh water prawn, *M. malcolmsonii* when exposed to Dichlorvos. There was a decline in the total protein as well as the enzymes, acetylcholine esterase and alkaline phosphatase in the various tissues. *P. vannamei* exhibits immunodepressioin on exposure to the fungicide propiconazole (Mũnoz *et al.*, 2000). It decreased the oxidative metabolism of haemocytes. Although an initial increase in the respiratory burst was seen, it subsequently decreased with increasing dose.

Joshi and Kumar (2001) have studied the effect of pesticides monochrotophos, aldrin, and carbaryl on the acid and alkaline phosphatase activity of the freshwater crab, *Paratelphusa masoniana*. Acid phosphatase activity increases under sub-lethal and lethal concentrations of the pesticides, but alkaline phosphatase is inhibited by the pesticides.

Bhavan and Geraldine (2004) have investigated the effect of the pesticide Endosulphan on acid and alkaline phosphates activities in the prawn, *M. macolmsonii*. The authors report that the enzyme level in some of the tissues, including haemolymph, is lower than that of the control.

2.2.2.1.2. Other organic pollutants

Smith and Johnston (1992) investigated the effect of polychlorinated biphenyls (PCBs) on the immune system of *Crangon crangon*. PCB 77 and PCB

15 are two structurally different congeners. Of these, PCB15 decreased the total haemocyte count of the animals.

Smith *et al.* (1995) has also observed a difference in various haemolymph factors of *C. crangon* exposed to contaminated harbour dredge-spoil. The shrimp exposed to contaminated dredge-spoils that contain polychlorinated biphenyls (PCBs), polynuclear hydrocarbons and heavy metals, showed variation in the immunity indices, especially the total haemocyte count. The animals displayed elevation in recoverable haemolymph volume and reduction in the blood cell phenloxidase activity. These results indicate a compromise in the immune capability and clotting nature. The total haemocyte count and the recoverable haemolymph volume showed marked difference in the animals exposed to dredge-spoil at a level as low as 5%, which indicates the effect on the host immune system.

2.2.2.1.3. Heavy metals

Heavy metals occur naturally in the environment. Many of them are essential for the normal metabolism of organisms. Under natural conditions, rivers are the main inputs of metals into coastal regions (Bryan, 1984). With the escalation of industrial activity, their concentrations in the aquatic environment have increased to dangerous levels. Anthropogenic activities are the major cause of heavy metal pollution in aquatic environments.

The pollution by heavy metals has always received widespread attention. This is so, because in addition to their persistence and toxicity, they have a tendency to bind onto particulates in soil and water. As potential hazards, especially in estuaries and coastal regions, they are second only to pesticides (Kinne, 1984). Heavy metals and their residues accumulate in animal tissues resulting in biomagnification at different trophic levels. This is especially true in the case of more abundant metals like copper and zinc, which prove to be more dangerous to the aquatic life (Bryan, 1984).

High concentrations of cobalt-60 have been reported in the giant clam, *Tridacna* (Lowman, 1960). Zinc and copper were detected in high concentrations

in oysters dwelling in waters, contaminated with heavy metal-containing effluent waters which cause stress (Waldiclunch, 1974). Poor larval performance of *Ostrea edulis* larvae is reported due to heavy metal contamination (Elderfield, 1971). Zinc is known to affect *Crassostrea virginica* embryos (Calubrese, 1972; Calabrese *et al.*, 1973) and causes retardation of growth in *C. gigas* (Breston *et al.*, 1973).

Accumulation patterns of zinc and other metals in crustaceans have been widely documented (Bryan, 1984; Prosi *et al.*, 1983; Jennings and Rainbow, 1979; White and Rainbow, 1982; Weeks and Rainbow, 1991). Studies on their effects on the physiology and particularly the immune system are scarce. Truscott and White (1990) have investigated the effects of copper, mercury and cadmium in *C. maenas*. Mercury does not affect haemocyte number. Copper has no effect on recognition or phagocytosis of bacteria, but cadmium slightly suppresses the phagocytic activity of haemocytes. In *S. serrata*, acid and alkaline phosphatase increases in the haemolymph and other tissues when subjected to sub-lethal concentrations of cadmium chloride (Reddy and Bhagyalakshmi, 1994). It was suggested that this increase in the lysosomal enzymes may be for increasing the breakdown of phosphates to release energy as a result of the impaired ATP-ase system, during cadmium exposure. Viswanathan and Manisseri (1995) have conducted toxicity and histopathological studies to assess the harm due to zinc in *F. indicus*. The LC_{50} of zinc to *P. indicus* juveniles was 1668.16 ppb. Sub-lethal concentrations as low as 100 ppb and 300 ppb were destructive and deteriorative to the hepatopancreas and gills of the animals. The safe concentration of zinc predicted by Laboratory Fish Production Index (LFPI) for *P. monodon* ranges from 0.0527 to 0.1317 ppm.

According to Pipe and Coles (1995), increase in the total haemocyte count on exposure to pollutants is either because of proliferation of cells or by the migration of cells from tissues to circulation. Similarly, decrease in the haemocyte count may be due to lysis, diapedesis, less recruitment or movement of cells from circulation to tissues. They have observed an increase in the total haemocyte count of cadmium-exposed molluscs challenged with *Vibrio tubiashi*.

Pipe *et al.* (1995) have investigated the total haemocyte counts of *Mytilus galloprovincialis* from areas polluted with chromium, nickel and mercury. They observed an increased total haemocyte count in all the exposed animals. Certain metals at low concentrations stimulate the defense mechanisms, as in *Mytilus edulis* exposed to copper (Pipe *et al.*, 1999). In *C. virginica* from sites contaminated with copper, zinc and tin, haemocyte density was significantly high, may be for sequestration and detoxification of metals from the system (Fisher *et al.*, 2000).

Palaemon elegans are highly susceptible to mercury, followed by cadmium, copper, zinc and lead. Ablated animals are more sensitive than intact animals. Copper and zinc being essential metals, higher concentrations of these produce hyperglycemic effect, which may be considered as a tolerance adaptation (Lorenzo *et al.*, 2000). Yeh *et al.* (2004) have reported that the copper sulphate causes a depression in immune ability in *L. vannamei*. The total haemocyte count, phagocytic activity and the respiratory burst were less in animals exposed to 1 mg/l of copper ion, when compared to control. The studies also revealed that higher concentrations of copper at 20 mg/l cause an increase in respiratory burst and a decrease in the phenoloxidase activity of exposed animals. The housefly, *Musca domestica*, shows reduced immune response when intoxicated with various heavy metals like zinc, copper, lead and cadmium. But they also bind these metals, especially lead and cadmium to their exoskeleton, which are periodically shed (Borowska *et al.*, 2004).

2.2.2.2. Histopathology of animals exposed to pollutants

Aquatic life is threatened by pollution from various substances. Pollutants accumulate in many organs of the body and cause changes in their basic structure. Histology of organisms is of prime importance in monitoring the level and nature of pollution and its effects on organisms.

In invertebrates, hepatopancreas has a parallel role of vertebrate liver and is the main site of metabolism of petroleum hydrocarbons (North, 1967; Nelson-Smith, 1970). It also accumulates heavy metals as seen in isopods (Prosi *et al.*,

1983). Gills are the respiratory organs that are in direct contact with the medium, and often with the contaminants in the media thus showing striking changes during oil and heavy metal toxicity (Papathanassiou and King, 1983).

Musko (1984) has described the histopathological alterations in the amphipods after insecticide contamination. Chandy and Kolwalkar (1984) studied the toxic effects of crude oil on *Charybdis lucifera* and *S. serrata.* Rupture of gill lamellae due to heavy accumulation of haemocytes, fusion of lamellae and epithelial damage were evident. Zonal tubular damage was observed in the hepatopancreas of both the crabs. Shrinking of cells and a decrease in the number of certain type of hepatic cells were seen in *C. lucifera.* In *S. serrata,* exposure to crude oil produced a decrease in the number of fibrillar cells in the hepatopancreas.

Fenitrothion is a broad-spectrum organophosphate pesticide used extensively as a larvicide against mosquitoes. Studies on this pesticide confirm the effect of such chemicals on non-target organisms. This organophosphate pesticide causes breakage of cuticular lining, degeneration of respiratory epithelium and severe haemocytic congestion in the gill lamellae of freshwater shrimp, *M. kitnensis* (Pawar and Katdare, 1984).

P. monodon was exposed to high and low concentrations of the insecticide, Perfekthion (active ingredient: dimethoate) (Vogt, 1987). Histology of the exposed animals showed that low concentrations can cause heavy damage to the organs especially the hepatopancreas, even when there was no manifestation of this in the behavior of the shrimps.

Krishnaja *et al.* (1987) studied the effects of heavy metals, mercury, cadmium, lead, arsenic, and selenium on *S. serrata.* One month long exposure to mercury and cadmium resulted in degenerative changes in hepatopancreas and gills. Extensive cellular proliferation (hyperplasia) of the secondary lamellae with cyst formation and necrotic regions are seen in the gills of cadmium exposed crabs.

Exposure of *S. serrata* to sub-lethal concentrations of cadmium results in the thickening of the gill lamellae, together with increase and accumulation of

haemocytes. Cells were seen to infilter into the haemocoelic spaces of the gills (Narayanan *et al.*, 1990). Victor *et al.* (1990) investigated the effects of mercury in the freshwater prawn, *M. idea*. The prawns that are exposed to 1 μg/l of mercuric chloride over a 30-day period exhibited hyperplastic gill lamellae with infiltration of haemocytes. The haemocytes are released into the inter-lamellar spaces through necrotic regions and they then cover the entire gill lamellae.

Lignot *et al.* (1997) have observed haemocytic congestion or thrombosis, lamellae necrosis and other alterations of gills and epipodites in *P. japonicus* exposed to lethal and sub-lethal doses of the organophosphorus insecticide, fenitrothion.

2.2.3. Salinity variation

Salinity of the surrounding medium is an important factor. Maximum growth of an organism occurs in an iso-osmotic media, because the animal would be spending minimum amount of energy for osmotic regulation. Fluctuations in salinity result in a deviation from this comfortable state, which may be detrimental to the organisms, especially at times of infections.

Gilles (1977) investigated the protein concentrations and patterns in the crustaceans *E. sinensis*, *C. maenas*, and *A. fluviatilis* reared in mediums of saline water and fresh water. It is seen that, there is an increase in protein concentration and copper containing protein fractions in animals reared in low saline medium. Boone and Schoffeniels (1979) reports that the production of haemocyanin increases in *C. maenas* maintained in low saline waters. Stress from viral infections during conditions of high salinity cause growth retardation (Bray *et al.*, 1990). Salinity with pH influences the growth as well as the osmoregulation of *C. maenas* (Allan and Maguire, 1992). Salinity with temperature influences survivability as observed in *P. chinensis*. In this animal, combinations of low salinity and temperature result in low survival (Chen and Lin, 1998). There is no change in the plasma protein concentration in *F. californiensis* with salinity reduction. But the total haemocytic prophenoloxidase increases with increasing salinity (Vargas-Albores *et al.*, 1998).

A significant difference was seen in the haemocyte numbers of *F. paulensis* maintained at different salinities (Le Moullac and Haffner, 2000). There was a reduction in the total haemocyte count with decreasing salinity of the medium. The count increased after wards. The total protein concentration of the animals decreased with salinity especially during the first week. The phenoloxidase activity decreased with salinity (Perazzolo *et al.*, 2002). Wang and Chen (2005) investigated the immune response of the white shrimp, *L. vannamei*, at different salinity levels. The authors reported a drop in the various immunological factors, namely, total haemocyte count, phagocytic activity and phenoloxidase activity, in animals reared at low salinities of 5 and 15 ppt.

2.2.4. Acclimation

A comparison between freshly caught *L. setiferus* males with those maintained in the laboratory for 7 days at two different temperatures have been examined by Sànchez *et al.* (2001) in connection with male reproductive melanization syndromes. A reduction was seen in total haemocytes, granular and semigranular cells in the acclimatized shrimp. An increase in the phenoloxidase activity was observed in the acclimatized animals, compared to the freshly captured ones. Other components that reduced were triglycerols, proteins, and cholesterol.

2.2.5. Moulting

The total haemocyte count and the diffrential haemocyte count showed variation during the moult cycle of the tiger shrimp, *P. japonicus* (Tsing *et al.*, 1989). There was a fall of 50% in the total circulating cells before moulting started. Le Moullac *et al.* (1997) reported differences in total cell count and in percentage of different cells in *L. stylirostris* during the moult cycle. It is seen that in *L. stylirostris,* the change in total haemocyte count is mainly due to the change in the percentage of hyalinocytes. The low phenoloxidase activity in crayfish during the premoult stage may be due to the increased amount of

inhibitors produced at this stage (Hergenbahn and Söderhäll, 1985; Johansson and Söderhäll, 1989).

2.2.6. Immunostimulants

Progress has been made in the prevention and treatment of diseases of cultured aquatic organisms. Today, various products are used for prophyalaxis against diseases from larval to grow out stages of cultured animals. Cell wall preparations from *Bifidobacterium thermophilum*, *Brevibacterium lactofermentum*, different *Mycobacterium* sp., *Norcardia rubra*, *Propionibacterium* sp., *Streptococcus* sp., *Vibrio* sp., fungus like *Schizophyllum communae* and yeast, *Saccharomyces cerevisiae*, and algal derivatives such as laminarin are used as immunostimulants. The active principles of immunostimulatory cell wall preparations are various muramylpeptide fragments, polysaccharides, lipopolysaccharides, lipopeptides, acyl oligopeptides and specific bacterial peptides (Duffus *et al.*, 1982) that increase the immune reactions of the animals (Devaraja *et al.*, 1998) thereby increasing their capability to eliminate pathogens (Vici *et al.*, 2000). Commercial immunostimulants are also available incorporating these active principles.

2.2.6.1. Cellular Factors

Zymosan is a purified cell wall extract from the fungus, *S. cerevisiae*. It contains the glycoprotein β-1, 3, glucan. Laminarin is a β-1, 3, β-1, 6, glucan from the algae *Laminaria hyperborea*. Unestam and Söderhäll (1977) observed that phenoloxidase from the crayfish, *A. astacus* is activated by the zymosan extract even up to a dilution of 10^{-11} M. Laminarin also activates phenoloxidase in crayfish serum. But a higher concentration of this glucan shows an inhibitory effect. In *A. astacus* and *Pacifastacus leniusculus,* these polypeptides elicit two important defense functions, activation of prophenoloxidase in haemocytes and cuticle and induction of a clotting process followed by phenoloxidase attachment to foreign surfaces (Söderhäll, 1981). Smith and Söderhäll (1983) found that pre-incubation of the haemocyte lysate of shore crab, *C. maenas* in laminarin,

enhances the activity of prophenoloxidase at least fourfold. A similar, but, weaker activation was achieved with zymosan. It was observed that the haemocyte count was also affected by glucans.

Smith *et al.* (1984) carried out a study to compare, *in vitro,* haemocyte response of *C. maenas* to a range of structurally different glucans. After injection of β-1, 3, glucan, a marked reduction in the number of circulating haemocytes concomitant with the formation of discrete clumps of haemocytes in the gills was observed.

The survival of cultured kuruma prawns was increased by the treatment with formalin-killed *Vibrio* sp., by injection, immersion, and spray techniques. The blood homogenate from immunized shrimps enhanced phagocytosis and other cellular defense mechanisms like nodulation and encapsulation by the hemocytes in untreated shrimp (Itami *et al.*, 1989). Oral administration of Schizophyllan, a β-1, 3 glucan from *Schizophyllan communae*, increases phagocytic activity in *P. japonicus*. The blood homogenate of such shrimps are able to activate phagocytosis of normal granulocytes (Itami *et al*, 1994). Takahashi *et al.*, (1995) has also mentioned the importance of oral administration of immunopotentiators like β-1, 3, glucan and peptidoglycan that increases the phagocytic activity in shrimp.

The production of reactive oxygen radical during phagocytosis increases with the immersion of shrimps in heat killed *Vibrio* antigen, yeast, β-1, 3, glucan and yeast cell wall zymosan. The result seems to be time dependent. The strongest production of O_2^- is detected at 3 hours in glucan treated shrimp and at 6 hours in *Vibrio* and zymosan treated shrimps (Sung *et al.*, 1996).

Du *et al.* (1997) have measured the phagocytic activity of the shrimp, *P. chinensis*. Acridine orange method (AO) was used to study both the phagocytosis and bacteria-killing simultaneously. With this method, they tested the effects of 4 imunopotentiating products, Zhenong I, Huzhou I, Huzhou II and Huzhou III, on the phagocytic activity and killing of *Staphylococcus aureus* by the shrimp haemocytes. The results showed that these four products could increase the

phagocytic activity of haemocytes and phenoloxidase and bacteriolytic activities in haemolymph.

P. japonicus given peptidoglycan derived from *B. thermophilium*, through feed showed resistance to *V. penaeicidia* and white spot syndrome baculovirus. This is suggested to be due to the increased phagocytic activity of granulocytes of treated shrimps (Itami *et al.*, 1998).

Sung and Sun (1999) has studied the effect of formalin-inactivated *V. vulnificus* on lysosomal enzymes in the haemocytes: acid phosphatase, alpha-naphthyl acetate esterase and β-glucuronidase in *P. monodon* and *M. rosenbergii*. The inactivated bacterium increased the acid phosphatase, α-naphthyl acetate and β-glucoronidase in the haemocytes.

Phagocytic activity and phenoloxidase activity are increased by polypeptides from algae. Polypeptides from *Pantoea agglomerans* in *P.japonicus* (Takahashi *et al.*, 1999) and PV911, an algal polysaccharide from *Cordyceps militaris* in *P. chinensis* (Jiang *et al.*, 1999) are known to increase the phagocytic activity. All the three haemocytes of *M. rosenbergii* were capable of phagocytosis, when treated *in vitro* with lipopolysachharide from *Escherichia coli*. The granulocytes and semigranulocytes engulfed zymosan particles, whereas, hyalinocytes showed only attachment to these particles. But all three-cell types were able to engulf the smaller sized fluorescent-labeled beads (Sung *et al.*, 2000). This lipopolysachharide greatly alter the haemocyte size and haemocyte morphology by increasing the stretching of pseudopodia and it also increases the number of engulfed particles per phagocyte. The percentage of hyalinocytes positive for acid phosphatase and α-naphthyl acetate esterase is more than that of semigranulocyte and granulocyte positive for these enzymes.

The probiont bacterium, *Bacillus* S11, given to *P. monodon*, in two 90-day culture trials, increased the total circulating haemocyte number. The total haemocyte counts showed greater values at day 60, when compared to day 30 in the probiont administered animals. This subsequently decreased by day 90. A decrease in the total haemocyte number is seen on challenge with *V. hareyi* in both the probiont and control animals. However, the animals administered with

bacteria showed an increase in the phagocytic abiliy. The value for phagocytosis is greater at day 60 than at day 30. Here also, by day 90 the value decreased (Rengpipat *et al.*, 2000). Aqua-mos, a commercial product that is a mannan-oligosaccharide extracted from *S. cereviae* cell wall, activate super-oxide anion (O_2^-) production in the haemocytes of *L. vannamei* (Motte *et al.*, 2001). Three strains of bacteria, *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 were isolated from the gut of *L. vannamei*. *Vibrio* P62 showed probiotic properties and *Bacillus* P64 had both probiotic and immunostimulatory features. The total haemocyte count of *L. vannamei* treated with these three strains of bacteria did not change. The hyalinocyte population in the *Bacillus* treated animals significantly increased, suggesting an increase in the circulation of young and immature cells and an enhanced activity of the haematopoietic tissue (Gullian *et al.*, 2004). Total haemocyte count of *P. monodon* fed with yeast-extract coated feed was higher than the control, which were fed with non-coated feed. The percentage of granular cells in the haemolymph was also higher (Sritunyalueksana *et al.*, 2005). *L. vannamei* given 4 to 6 µg/L of hot-water extract of *Gracilaria tenustipitata* showed increased total haemocyte count, phenoloxidase activity phagocytosis and respiratory burst.

2.2.6.2. Humoral factors

Phenoloxidase in the haemocyte lysate of *P. monodon* is significantly enhanced on immersion treatment with immunostimulants. The tiger shrimp were treated with three immunostimulants viz. heat killed *Vibrio*, *β*-1, 3, glucan and zymosan extracted from *S. cerevisiae*. All these immunostimulants increased phenoloxidase activity from 5 minutes to 1 day, when compared to control animals. The results showed that intra-haemocytic phenoloxidase production increases by immunostimulant treatment by immersion, with the greatest activity occurring at 3 hours, and lasting for 3 days (Sung *et al.*, 1996).

Chitin has been used as an additive in the feed of *P. japonicus* to evaluate its effectiveness on the growth and immunocompetance (Chang *et al.*, 1997). Shrimps that were fed with feed containing 400, 600, 800, and 1000 mg/kg diet

of chitin showed significant increase in growth and phenoloxidase activity. When chitin was given at a dose of 400 mg/kg diets for different time periods, protective effect was highest after 10-day continuous administration and lasted for at least five more days.

Jiang *et al.* (1999) have observed that algal polysaccharide PV911 and *Cordyceps militaris* polysaccharide increased the phenoloxidase activity of the serum in *P. chinensis*.

Phenoloxidase, agglutinating and antibacterial activities occurr naturally in the shrimp, *P. monodon*, but vary considerably amongst the individuals. When peptidoglycan, lipopolysaccharide and laminarin are added to haemolymph lysate supernatent fraction, the former two are able to raise phenoloxidase activity, *in vitro* and *in vivo*. Laminarin fails to elicit any defense reaction (Sritunyalucksana *et al.*, 2005).

A crude extract isolated from mango leaves (*Mangifera indica*) given to *P. monodon*, along with their diet, increased the phenoloxidase activity of the haemolymph (Chamanon *et al.*, 1999). The activation of prophenoloxidase to phenoloxidase takes at least three steps: recognition of β-1, 3, glucan, activation of serine protease, and processing of prophenoloxidase. This process of activation has been shown in *P. japonicus* with laminarin. It induces phenoloxidase activity in haemocyte lysate supernatent and not in purified phenoloxidase and prophenoloxidase. Laminarin also enhances activity of protease in the lysate supernatent. Serine protease inhibitors inhibit both the reactions (Adachi *et al.*, 1999).

Haemocytes of *M. rosenbergii* treated with lipopolysaccharide from *E. coli* showed increased production of acid phosphatase and α- naphthylacetate estrase during phagocytosis (Sung *et al.*, 2000).

Glycoprotein isolated from *Flavobacterium odoratum* has activated the immune factors in serum of *P. clarkii*. The lectin, phenoloxidase, bacteriolysins and antibacterial factors in serum are higher in treated animals than control animals (Mo *et al.*, 2000). Immunoplus is a commercial formulation used as an immunostimulant. The total protein level of *M. rosenbergii* increased upto two

weeks of feeding immunoplus at 1g/kg of feed. Phenoloxidase, haemagglutination and lysozyme activities remain high up to three weeks of feeding (Kumari *et al.*, 2004).

The phenoloxidase activity of *P. monodon* increases with the addition of the probiont *Bacillus* S11 in their feed. The phenoloxidase activity increases upto the 60-day period after which, it decreases. After 10 days of feeding, the animals challenged with *V. harveyi*, shows increase in phenoloxidase activity (Rengpipat *et al.*, 2000).

Gullian *et al.* (2004) has isolated *Vibrio* P62, *Vibrio* P63, and *Bacillus* P64 from the hepatopancreas of *L. vannamei*. All the aniamals fed with these three bacteria, showed significantly increased phenoloxidase activity than the control, which indicates that the cells are strongly stimulated.

Material and Methods

3. Material and Methods

3.1. Experimental animals

Juveniles of *F. indicus* (7.23 ± 2.14 cm) were collected from a cooperative farm in Vypeen Island and brought to Central Marine Fisheries Research Institute. The animals were stocked in one ton fibre glass tanks at the rate of 30 numbers per tank. The tanks were provided with continuous aeration. Water salinity was maintained at 25 ppt. The animals were acclimatized to laboratory conditions for a minimum period of one week. Shrimp were fed with commercial pellet feed at the rate of 3% body weight, daily. Feacal matter and uneaten feed were siphoned out, and 75% of the water was replaced daily. Injured and weak shrimp were discarded.

3.2. Haematological studies of *F. indicus*

3.2.1. Cellular factors

3.2.1.1. Characterization of haemocytes

3.2.1.1.1. Light microscopy

The method followed was of Mix and Sparks (1980) with modifications. The animals were blotted dry of water. From each shrimp, 100 µl of haemolymph was withdrawn from the heart. A 2 ml syringe containing freshly prepared, cold, 10% seawater-formalin (1.4 ml), attached to 26 guage needle was used. The syringe was shaken and rotated gently and the contents transferred to an eppendorf tube. The sample was kept at 4°C for 1-3 hours for proper fixation. The haemolymph was centrifuged at 8000 rpm for 5 minutes at 4°C. The supernatant was decanted. The cell pellet was mixed with two drops of supernatant and thick smears were prepared on clean, grease-free glass slides. They were air dried, post-fixed in absolute methanol for 5 minutes, rinsed in distilled water and stained with dilute Wright's stain of pH 7.2 for 5-10 minutes. The scum was washed off with distilled water and the slides were dried. The smears were viewed under a light microscope. The cells were classified on the basis of presence or absence of granules and their staining reactions.

3.2.1.1.1.1. Cell dimensions

Measurements were made in Wright's stained smears. Triplicates of 200 stained cells from three slides were measured using a calibrated ocular micrometer. The cell size was determined by measuring the largest and shortest axis excluding pseudopodia. Nuclear size was estimated by measuring the largest and shortest nuclear diameter. Measurements were taken for all the cells.

3.2.1.1.2. Transmission electron microscopy

The method of Martin and Graves (1985) was modified for ultrastructural studies of *F. indicus* haemocytes. A 26-gauge needle attached to a 1 ml syringe was used to withdraw 0.1 ml haemolymph from the heart. The syringe contained 0.4 ml chilled fixative (3% glutaraldehyde in 0.1M sodium cacodylate buffer containing 12% glucose, pH 7.8). After thorough mixing, the sample was transferred to an eppendorf vial and immediately kept at 4°C for 2 hours. The fixed sample was centrifuged at 8000 rpm for 5 minutes at 4°C. The pellet was given three washes with 0.1 M sodium cacodylate buffer (pH- 7.8, containing 24% sucrose). It was post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at 4°C. The sample was centrifuged at 8000 rpm for 5 minutes and the pellet was washed three times in 0.1 M sodium cacodylate buffer (pH 7.8 containing 24% sucrose). Two percent agarose (HiMedia Ltd) gel was prepared in cacodylate buffer (pH- 7.8) containing 24% glucose. The molten gel was cooled to 60°C and added to the eppendorf vial in a water bath at 60°C. The sample was mixed thoroughly and allowed to solidify. The solidified gel was cut into tiny pieces of 1 mm thickness and dehydrated in an ascending series of acetone. The sample was further processed for electron microscopy. It was infiltered and embedded in Spurr's (1969) low viscosity resin. Thin sections of 60-90 nm were cut on an LKB-Ultramicrotome, stained with uranyl acetate and lead citrate and viewed in a Hitachi-H600 Transmission electron microscope.

3.2.1.1.3. Cytochemical analysis

Various cytochemical studies were conducted in the haemocytes of *F. indicus*. Cytochemical staining methods were employed to demonstrate carbohydrates, lipids and the enzymes prophenoloxidase, acid phosphatase and myleoperoxidase in haemocytes.

3.2.1.1.3.1. Periodic Acid-Schiff's (PAS) reaction

The presence of carbohydrates in the haemocytes of *F. indicus* was studied using the PAS method of Sanders (1974). Haemolymph was drawn into a 2 ml syringe containing citrate/EDTA buffer, pH 7.3. The cells were concentrated by centrifugation at 8000 rpm at 4^0 C for 15 minutes. Haemocyte smears were made on clean grease-free glass slides. These were fixed with methyl alcohol and incubated in 0.5% periodic acid for ten minutes. The smears were rinsed with distilled water and treated with Schiff's reagent for 15 minutes. After this, they were placed in 3 changes of sulphurous acid rinse (10 ml of 1N HCl in 210ml of 0.05% Na HCO_3) for 3 minutes each. The smears were left in running water for five minutes and rinsed in distilled water. They were stained with Harris haematoxylin for 30 to 45 seconds, after which washes were given in running water till the smears turned blue.

3.2.1.1.3.2. Demonstration of lipids

The method of Sanders (1974) was used to test the presence of lipids in *F. indicus* haemocytes. Air-dried smears were placed in formalin vapours for ten minutes. The fixed smears were rinsed in running water, after which they were layered with Working Sudan Black B solution for 1 hour. The smears were washed in running water for 2 to 3 minutes. After blotting dry, they were counterstained with Maygunwald-Giemsa stain and viewed under a microscope.

3.2.1.1.3.3. Prophenoloxidase activity

Haemocyte smears were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH- 7.4) for 1 minute at 4°C and washed three times (for 15

minutes each) with 0.1 M sodium phosphate buffer (pH- 7.4). The slides were then incubated in 0.1% L-dopa (dihydroxyphenyl alanine) prepared in 0.1 M phosphate buffer with 2% NaCl for 90 minutes at 30°C (Smith and Soderhall, 1983) and counterstained with dilute Giemsa.

3.2.1.1.3.4. Acid phosphatase activity

As per the method described by Sanders (1974), air-dried smears were fixed in formalin-acetone (20% formalin in 50% acetone) for 1 minute at 0°C and rinsed in running water. The fixed smears were incubated at 37°C for 1 hour in a freshly prepared and filtered acid phosphatase substrate solution containing Fast Blue BBN. The slides were then rinsed with distilled water, air dried and counterstained with neutral red solution for 3 minutes. Control slides were also prepared by incubation with stock solution without Fast Blue BBN.

3.2.1.1.3.5. Myleoperoxidase activity

Air dried smears were fixed in 10% alcoholic formalin for 60 seconds, rinsed in distilled water for 15-20 seconds. Slides were then incubated in myleoperoxidase incubation mixture (100 ml of 30% ethyl alcohol, 0.3 g of benzidene dihydrochloride, 1 ml of 0.132 M zinc sulphate, 0.7 ml of 3% hydrogen peroxide, 1.5 ml of 1N sodium hydroxide and 0.2 g of Safranin O) according to the method of Sanders (1974) for 30 seconds, washed briefly in running tap water, dried and counter stained with Giemsa.

3.2.1.2. Total haemocyte count (THC)

Twenty micro litre of haemolymph was drawn from the heart of the shrimp with a 26 gauge needle into a 2 ml syringe containing 1 ml cold 10% seawater formalin (Mix and Sparks, 1980). After thorough mixing it was transferred to an eppendorf vial and kept at 4°C for 1 hour. The cell suspension was loaded in to a haemocytometer and the total haemocyte count was determined per millilitre of haemolymph (Perazzolo and Barracco, 1997).

3.2.1.3. Differential Haemocyte Count (DHC)

From each animal, 20 µl haemlymph was drawn into a syringe with 1 ml fixative (Mix and Sparks, 1980) and the contents stored for 1 hour at 4°C. This was centrifuged at 8000 rpm for 5 minutes. From the concentrate, thick smears were prepared on glass slides and stained with dilute Wright's stain of pH 7.2. From each slide, 200 cells were observed under oil emulsion objective (1000X) and the percentage of each cell calculated.

3.2.1.4. Phagocytic activity of haemocytes

Fifty micro litre of haemolymph was drawn into 1 ml of anticoagulant (cold, freshly prepared citrate/EDTA buffer, pH-7.3 (Le Moulac *et a.l*, 1997) and K-199 medium, pH-7.3 (Itami *et al.*, 1994) taken in the ratio 1: 1. The cells were separated from the haemolymph by centrifugation at 8000 rpm at 4°C for 10 minutes and washed three times with the medium. The cells were supplemented with fresh K-199 medium, mixed gently and layered on clean glass cover slips. These were incubated at 25°C for 30 minutes and the cells were allowed to adhere to the coverslips. The non-adhered cells were washed off gently with K-199 medium. Then the cover slips were layered with killed yeast cells (1×10^8 cells/ml) suspended in K-199 medium. The layered cover slips were incubated at 25°C for 90 minutes, washed gently with medium and fixed with absolute methanol for 5 minutes. They were washed with medium and stained with Wright's: Giemsa (1:1) stain, pH-6.8. The stain was washed off with distilled water, dried and mounted on glass slides. Two hundred cells were counted from each slide and the percentage phagocytosis calculated as follows,

Percentage phagocytosis = (number of haemocytes ingesting yeast cells/total number of haemocytes observed) × 100.

3.2.1.4.1. Nitro Blue Tetrazolium reduction assay

The production of O_2^- during oxygen burst was detected using Nitro Blue Tetrazolium reduction assay. The method followed was of Anderson *et al.* (1991) with modifications. Phagocytosis was performed according to the above

procedure. The excess yeast on the cover slips was washed off with PBS (pH-7.4) and the cover slip air-dried. The cover slip was covered with filtered NBT solution (0.2% in pH-7.4 PBS) for 30 minutes and kept in a humid chamber. The excess stain was decanted off, washed, air dried and fixed in methanol. It was mounted in DPX on a clean glass slide and observed under the microscope.

3.2.2. Humoral factors of the haemolymph

The parameters studied were the serumprotein profile of serum *i.e.,* total serum protein concentration, the serum polypeptide profile, haemagglutinating activity and activity of different enzymes *viz.* phenoloxidase, acid phosphatase and alkaline phosphatase.

3.2.2.1. Preparation of serum

Haemolymph drawn from the heart of shrimp without anticoagulant was quickly transferred to an eppendorf vial and allowed to clot at 4°C for 4 hours. The serum was separated from the clot by centrifuging at 8000 rpm at 4°C for 10 minutes and used for studying various humoral factors.

3.2.2.2. Protein profile of serum

3.2.2.2.1. Total serum protein concentration

The total protein concentration was determined using the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard.

3.2.2.2.2. SDS-PAGE of serum

The serum proteins were separated by SDS-PAGE by the method described by Laemmli (1970). Electrophoresis was carried out on 10% separating gel. One microlitre of serum sample was diluted 100 times with double distilled water. The samples were mixed with equal volumes of sample buffer, boiled for 5 minutes and loaded in the wells along with standard protein marker (Genei, India). Electrophoresis was carried out at 140v for 5-6 hours and the gel was stained with Coommasie brilliant blue.

3.2.2.3. Haemagglutination

Haemagglutination was performed in 96 well 'v' bottom micro titer plates following the method of Imai *et al.* (1994). Serial double dilutions of the serum (25 µl) were made using 0.85% Na Cl containing 5 mM CaCl as diluent. Haemagglutinating activity was carried out using 2% (v/v) chicken RBC suspension (25 µl) in phosphate-buffered saline. The plate was allowed to stand for 1 h at room temperature and for 24 h at 4^0C. The agglutination titer was recorded as the reciprocal of the maximum dilution giving positive agglutination.

3.2.2.4. Enzyme profile of haemolymph

3.2.2.4.1. Phenoloxidase activity

The method of Preston and Taylor (1970) with modifications was followed. To 2 ml of 0.01 M dihydroxyphenyl alanine (DOPA) in Tris-HCl buffer (0.05M, pH 7.5), 0.2 ml of enzyme source was added. Two hundred micro litre of sodium dodicyl sulphate in 0.05M Tris buffer (mg/ml) was added as activator. The increase in O.D. of the sample was recorded up to 3 minutes for every 30 seconds interval at 420 nm. The results were expressed as enzyme units (0.001 change in O.D./minute/mg of protein). The total protein was estimated using the method of Lowry *et al.* (1951).

3.2.2.4.2. Acid phosphatase

The method followed was of Varley (1980). Ten microlitre of serum was diluted 10 times with phohosphate buffered saline (pH 7.3). It was allowed to react with 0.01 M disodium phenyl phosphate and citrate buffer in 1:1 proportion at a pH of 4.9. The phenol released was allowed to react with 0.6% of amino-antipyrene in 2.4% Ferricyanide solution in 0.5N sodium hydroxide and 0.5N of sodium bicarbonate. The O.D was recorded at 510 nm. The amount of phenol released per 100 ml serum was determinedfrom a standard curve usingknown amount of phenol. The results were expressed in KA units (mg phenol released/ 100 ml serum/ h).

3.2.2.4.3. Alkaline phosphatase

Twenty five microlitre of serum was diluted to 50 µl with phosphate buffered saline (pH. 7.3). The enzyme activity was estimated using a commercially available diagnostic kit (Sigma Diagnostics, India Ltd, Product No. 72011, according to King and King (1954). The O.D. was measured at 510 nm and enzyme activity was expressed in KA units.

3.3. Immunomodualtion in *F. indicus*

3.3.1. Exposure to *V. parahaemolyticus*

3.3.1.1. Estimation of sub-lethal dose

A pathogenic strain of *V. parahaemolyticus* species isolated from diseased shrimp was used for experimental infection. Dilutions in phosphate buffered saline with a cell count of 10^7cells/ ml, 10^6cells/ ml, 10^5cells/ ml, and 10^4/ ml was chosen to determine the 24 hour lethal concentration. For each concentration 30 shrimps were maintained in triplicates. The animals were injected intramuscularly with the respective dosages on the fifth abdominal segment. A bacterial count of 10^5/ ml was determined as the lethal dose.

3.3.1.2. Experimental infection

Seventy animals were maintained in 1 ton tanks, each for control and test groups. A bacterial cell density of 10^4cells/ml was used as the sub-lethal dose ($1/10^{th}$ of the lethal dose). Each shrimp was inoculated with 0.1 ml of the bacterial suspension in PBS on the fifth abdominal segment. The control group was injected with 0.1 ml of PBS per animal. 10 shrimps, each from test and control groups were sampled after 30 minutes, 3 hours, 1 day, 2 days, 3 days, 1 week and 2 weeks. Heamolymph collected from the heart was pooled. Shrimp samples were also fixed in Davidson's fixative for histological studies.

3.3.2. Exposure of *F. indicus* to organophosphate pesticide, nuvan

The organophosphate pesticide used for the study was dichlorvos, known by the trade name nuvan. Initial experiments were conducted to determine the LC_{50} of the compound.

3.3.2.1. Estimation of LC_{50} of Nuvan for *F. indicus*

The commercial preparation of dichlorvos, nuvan, marketed by Syngenta India Ltd., was used. The concentration of active ingredient was 1000 mg/ ml. The 96 h LC_{50} of nuvan for the experiment was determined as per the method of Reish and Oshida (1987). One millilitre of nuvan was added to 100ml of distilled water, so that 1 ml of the solution contained 10 mg of nuvan. The concentrations selected for calculating 96 h-LC_{50} were 0.2, 0.1 and 0.05 ppm of nuvan. Ten animals were kept in 50 liters of 25 ppt seawater for each test concentration. They were maintained in triplicates for a week. A control was also maintained in triplicates. Complete water exchange was made everyday of the experiment and the chemical concentration maintained. The moralities were plotted on a logarithmic graph and 50% of mortality concentration was identified from the graph. Thus the 96-hour LC_{50} was found to be 0.1 ppm.

3.3.2.2. Experimental exposure

The animals were given 1 week of acclimatization. The animals were exposed to nuvan at 0.02 ppm, 0.05 ppm and 0.08 ppm concentration, respectively. Each dose was tested in triplicates reared as groups of 10 animals. The control group was also maintained. Twenty-five percentage and 75 % water exchange was made on alternate days, during which the concentration of the chemical in the water was maintained. The animals were given pelleted feed at 3% body weight, twice a day. The experiment period lasted 2 weeks. Samples were taken after two weeks. Pooled heamolymph was used for analysis. For histological studies, samples were fixed in Davidson's fixative.

3.3.3. Exposure of *F. indicus* to heavy metal, zinc

To study the effect of exposure to the heavy metal zinc in shrimps, animals were exposed to sub-lethal concentrations of zinc. As per the report of Viswanathan and Manisseri (1995) the 96 h LC_{50} of zinc for *F. indicus* juveniles is 1668.1 ppb, *ie*; 1.6681 ppm. Hence a sub-lethal concentration of 0.1ppm was selected as the test concentration.

3.3.3.1. Preparation of heavy metal salt solution

The chemical used for the experiment was zinc sulphate (ZnSO4. $7H_2O$, M.W. – 287). A stock solution of 3 ppm zinc was prepared by dissolving 6.63 g of zinc sulphate in 500 ml of distilled water. A calculated amount was added to the experimental tubs, containing known volume of seawater, to get the desired concentration of 0.1 ppm zinc in the medium.

3.3.3.2. Experimental design

F. indicus juveniles were stocked at a rate of 6 animals per 30 l of 25 ppt diluted seawater. Triplicates were maintained. The tubs were provided with ample aeration. A control group maintained in triplicates was also kept. The animals were fed on commercial pellet feed *ad libitum*. Eighty percent of the rearing medium was renewed every day and the required amount of heavy metal maintained. The experimental period was for 2 weeks. At the end of two weeks, the pooled haemolymph from the animals of each replicate was analyzed. On termination of the experiment shrimp samples were fixed in Davidson's fixative for histology studies.

3.3.4. Exposure of *F. indicus* to salinity variation

Juveniles of *F. indicus*, after an acclimation period of 5 days, were transferred to experimental tanks (100 l) with continuous aeration. Salinity levels of 3 ppt, 10 ppt and 25 ppt were chosen for the experiment. Ten animals per tank were maintained in triplicates in diluted seawaters of respective salinities. The animals were slowly acclimatized to the respective salinity. All the groups were

fed with a commercial pellet feed at a rate of 3% average body weight. Fecal matter and food waste were siphoned out every day. A water exchange of 50%, and complete water exchange on alternate days was made during the entire experimental period of two weeks. The haematological parameters were analyzed from the pooled haemolymph.

3.3.5. Effect of immunostimulant on the defense factors of *F. indicus*

The animals (6.5±2.1 g) were stocked at a density of 45 animals in 1 ton capacity tanks after an acclimation period of 1 week. The salinity of the tank water was maintained at 25 ppt and continuous aeration was provided. Control groups were maintained under the same conditions. Shrimp were fed with commercial pellet feed. The feed for the test group was incorporated with 'Allways", a commercial immunostimulant (Matrix BioScience India Ltd) containing β- 1, 3glucan, at a rate of 0.002g/1000g feeds. The test animals were fed at a rate of 2% of the body weight per day. The control animals received the same feeding regime, but without the immunostimulant. The tanks were cleaned of uneaten feed and faecal matter, and the water exchanged, every day. The experiment was carried out for a period of one month. Shrimp samples were collected weekly intervals. The pooled haemolymph was used for analysis. Samples were fixed in Davidson's fixative for histological analysis.

3.4. Statistical Analysis

The soft wares, Windows SPSS.10. and Systat 7.0.1 were used for statistical analyses. The results were analyzed using Analysis of Variance technique at 5% level of significance. The means were compared using Duncans multiple range test. Two sample t-test was used to test the significance for zinc exposed animals as also between the treatment means in bacterial and immunostimulant treatments.

3.5. Histology

The cephalothorax of *F. indicus* was dissected, fixed and processed according to the procedure of Bell and Lightner (1988). Specimens were fixed in Davidson's fixative and transferred to 50% ethyl alcohol after 24 hrs. The tissues were decalcified with 10% EDTA, dehydrated in ascending series of alcohol, cleared in xylene and embedded in paraffin wax (HiMedia,ltd). Trimmed blocks were cut into thin sections of 5-6 microns and mounted on clean, grease-free glass slides and mounted in DPX. The sections were stained using Haematoxylin and Eosin and examined under Leica (DMLS 60) trioccular photomicroscope and photomicrographs were taken. Gills, heart, hepatopancreas, haeamatopoietic tissue, and antennal gland of the samples were studied.

Results

4. Results

4.1. Haemolymph factors of *F. indicus*

4.1.1. Morphological characterization of haemocytes

Light microscopic examination of haemolymph smears of *F. indicus* revealed mainly three classes of haemocytes. Based on the size of cells, the absence or presence of cytoplasmic granules and staining properties of the granules under Wright's stain, these were classified into hyalinocytes, small granule haemocytes and large granule haemocytes (Plates 1& 2).

Hyalinocytes were small (5.69 ± 1.2 × 4.9 ± 0.9μm) with a nucleus (3.99 ± 1.0 × 3.49 ± 1.0 μm) occupying most of the cell volume. The small amount of cytoplasm along the periphery was devoid of distinct granules (Plates 1&2). In contrast, the small granule haemocytes were larger (9.59 ± 1.8 × 6.13 ± 1.2 μm) with abundant cytoplasm. The nucleus of these haemocytes (5.55 ± 0.9 × 4.29 ± 1.0 μm) was comparatively smaller. Small, but distinct basophilic granules were observed in the cytoplasm of the cells (Plate 2). In large granule haemocytes the distinguishing feature was the presence of large, distinct, highly acidophilic cytoplasmic granules. This type of haemocyte was large with a mean size of 11.42 ± 2.4 × 6.87±1.2 μm. The nucleus (4.55 ± 1.4 × 3.12 ± 1.3 μm) was lobed in majority of the cells (Plate 1). In some haemocytes, basophilic and acidophilic granules together were noticed in the cytoplasm.

Ultrastructural studies also confirmed the presence of three forms of haemocytes. The hyalinocytes displayed a high nucleo-cytoplasmic ratio and contained more heterochromatin than euchromatin. Electron dense cytoplasmic granules were absent in this group. Mitochondria were present occasionally. Psuedopodia and vacuoles were present in some cells. Small electron lucent granules were observed in some of the hyalinocytes (Plates 3-5).

The cytoplasmic granules of the small granule haemocytes were small and spherical, but electron dense. The abundant cytoplasm of the cells was traversed by a well developed rough endoplasmic reticulum. A number of mitochondria, free

ribosomes and Golgi bodies were also observed in the cell. The nucleus of these cells was small and lobed and the amount of euchromatin was more than heterochromatin (Plate 4 & 6). Some of the cells contained in addition to small granules, a number of large granules also (Plates 7& 8).

The cytoplasm of the large granule haemocytes was filled with large, electron dense granules. The number and shape of the granules were found to vary in these haemocytes. Other organelles observed were endoplasmic reticulum, occasional mitochondria and free ribosomes. Vacuoles and pseudopodia were also visible in the cells. The nucleus contained more heterochromatin than euchromatin (Plate 9-12).

4.1.2. Cytochemistry of haemocytes

4.1.2.1. Carbohydrates

Periodic Acid Schiff's (PAS) reaction revealed a limited number of haemocytes positive for the presence of carbohydrates in the granules (Plate 13).

4.1.2.2. Lipids

Many of the haemocytes of *F. indicus* were positive to Sudan Black B staining. In some of the haemocytes, dark stained areas were noticeable along the periphery of the cell membrane, whereas in some other, discrete dark granules, positive for lipids were observed in the cytoplasm (Plate 14 &15).

4.1.2.3. Prophenoloxidase enzyme

A limited number of large haemocytes displayed intense staining reaction. There were numerous haemocytes showing moderate enzyme activity that were small in size. In both groups of haemocytes, enzyme activity was seen in the cytoplasm. Haemocytes showing negative reaction were also noticed (Plate 16).

4.1.2.4. Myleoperoxidase enzyme

Majority of haemocytes were positive to peroxidase enzyme assay, which was seen as dark blue colored granules in the cytoplasm. Haemocytes with negative reaction were also present (Plate 17).

4.1.2.5. Acid phosphatase enzyme

Acid phosphatase enzyme activity appeared as distinct blue granules in a small number of haemocytes. The number of such granules in a haemocyte was also less (Plate 18).

4.2. Haematological study of apparently normal *F. indicus*

4.2.1. Cellular factors

4.2.1.1. Total haemocyte count (THC)

The total haemocyte count of apparently normal *F. indicus* ranged from 1315 to 2431 $\times 10^4$ cells/ml of haemolymph with mean value of 1978.65 $\pm$ 428.46 $\times 10^4$ cells/ml (Table 1).

4.2.1.2. Differential haemocyte count (DHC)

The differential haemocyte count of the various haemocytes revealed that small granule haemocytes are the predominant form in *F. indicus*. The percentage of small granules ranged between 54% and 77%, with a mean of 67.66 $\pm$ 6.28 %. The mean percentage of hyalinocytes of normal animals was 22.92 $\pm$ 2.82 % and varied from 20% to 28 %. The percentage of large granule haemocytes ranged from 3% and to 11%, with a mean of 8.35 $\pm$ 2.78% (Table 1).

4.2.1.3. Phagocytic activity

In vitro phagocytic assay of the haemocytes of *F. indicus* with yeast cells as substrate revealed a mean percentage phagocytosis of 53.15 $\pm$ 8.37%. The number of cells exhibiting phagocytic activity ranged from 30% to 67% (Plates 18&20).

4.2.1.3.1. Oxygen radical formation

Nitro blue tetrazolium (NBT) reduction assay of haemocytes displayed a strong reaction in the form of large number of discrete, dark blue formazan granules. These granules were seen at the sites of phagocytosed yeast cells demonstrating the formation of oxygen radicals during phagocytosis (Plate 21)

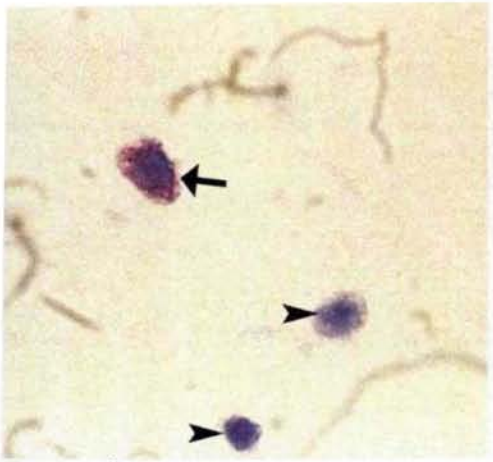

Plate 1.Hyalinocyte (arrow head) and large granule haemocyte (arrow) of *F. indicus*. Note the acidophilic granules of the large granule haemocyte. Wright's stain. 1000X.

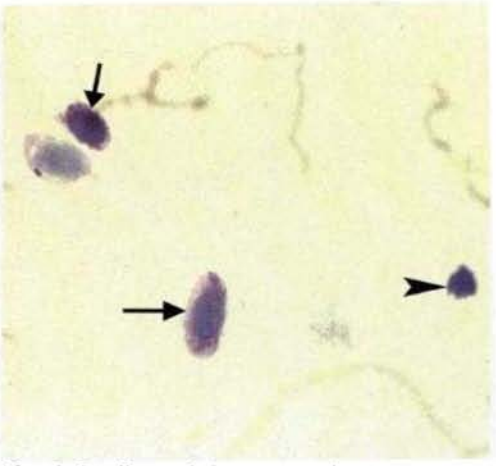

Plate 2. Small granule haemocyte of *F. indicus* (arrow). Note the presence of small, basophilic granules in the cytoplasm. A hyalinocyte can be seen nearby (arrow head). Wright's stain. 1000X.

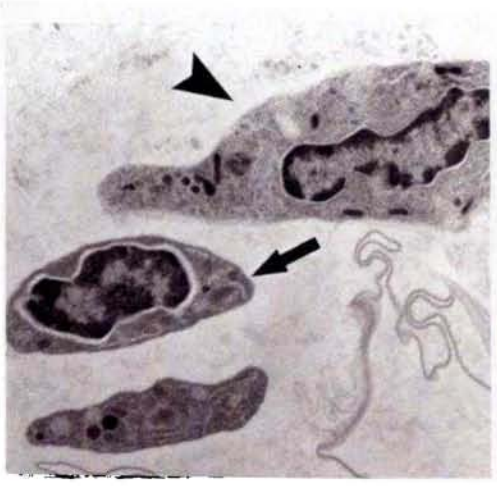

Plate 3. Electron micrograph of haemocytes of *F. indicus*. A hyalinocyte (arrow) and small granule haemocyte (arrow head) can be seen. 5000X.

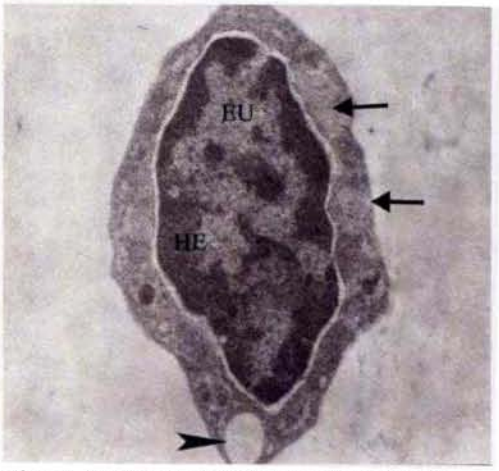

Plate 4. Hyalinocyte of *F. indicus*. Mitochondria (arrow) and vacuole (arrow head) are present in the cytoplasm. Note more of heterochromatin (HE) than euchromatin (EU) in the nucleus. 15000X.

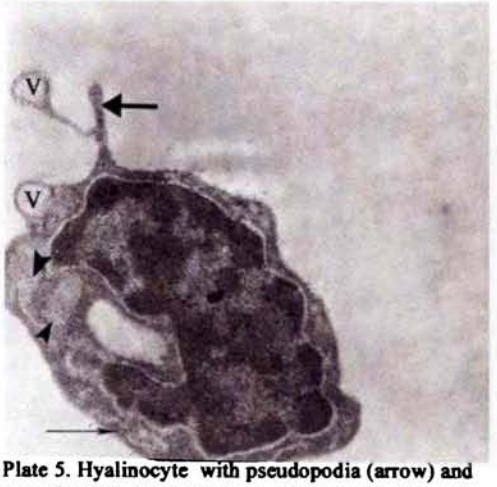

Plate 5. Hyalinocyte with pseudopodia (arrow) and vacuoles (V). The cytoplasm contains mitochondria (arrow head) and developing endoplasmic reticulum (line arrow). 12000X.

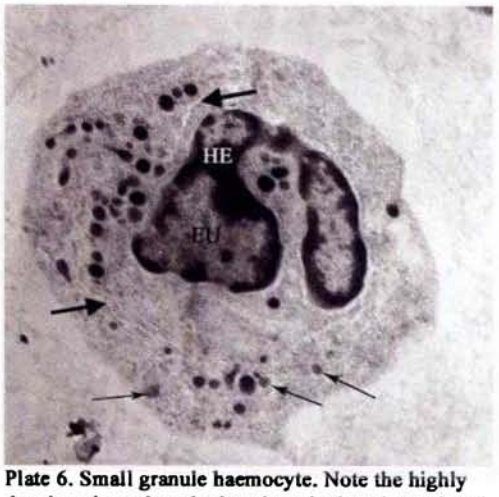

Plate 6. Small granule haemocyte. Note the highly developed rough endoplasmic reticulum (arrow) and numerous mitochondria (thin arrows) in the cytoplasm. Nucleus contains more of euchromatin (EU) than heterochromatin (HE). 6000X.

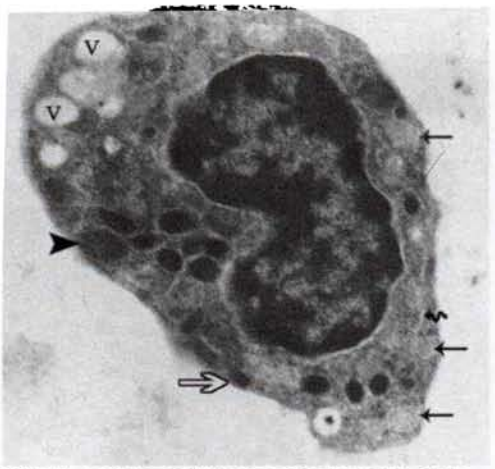

Plate 7. An intermediate stage of haemocyte. Note the presence of large (arrow head) and small (hollow arrow) electron dense granules, numerous mitochondria (thin arrow),endoplasmic reticulum (bent arrow) and vacuoles(V).10000X.

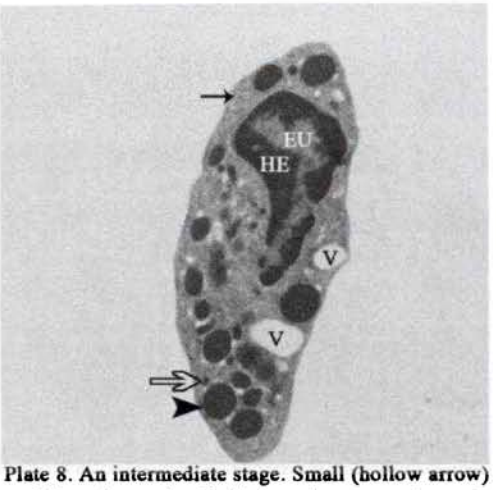

Plate 8. An intermediate stage. Small (hollow arrow) and large (arrow head) electron dense granules and vacuoles (V) can be seen. Mitochondria (thin arrow) are limited.10000X.

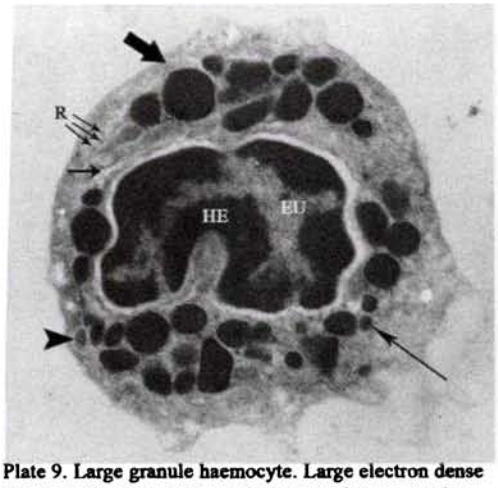

Plate 9. Large granule haemocyte. Large electron dense granules (block arrow), small electron dense granules (line arrow), endoplasmic reticulum (small arrow),mitochondria (arrow head) and free ribosomes (R) can be seen .Note more heterochromatin (HE) than euchromatin(EU). 12000X.

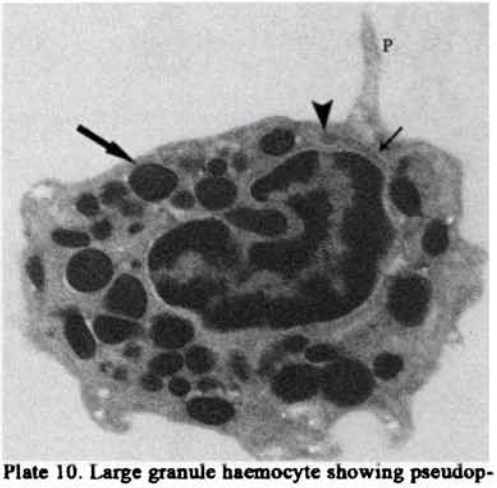

Plate 10. Large granule haemocyte showing pseudopodia (P). Large electron dense granules of different shapes can be seen (large arrow). Also seen in the cytoplasm are endoplasmic reticulum (arrow) and mitochondria (arrow head).12000X.

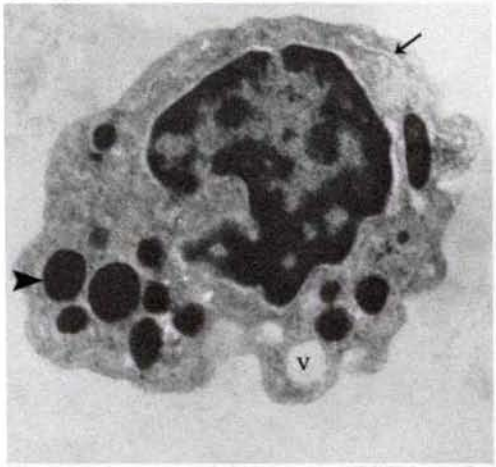

Plate 11. A large granule haemocyte with less number of large, electron dense granules (arrow head). Endoplasmic reticulum (arrow) and vacuoles (V) are also present. 12000X.

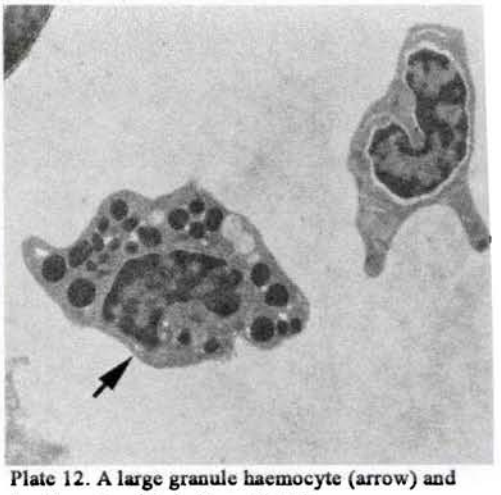

Plate 12. A large granule haemocyte (arrow) and hyalinocyte of *F. indicus*. 6000X.

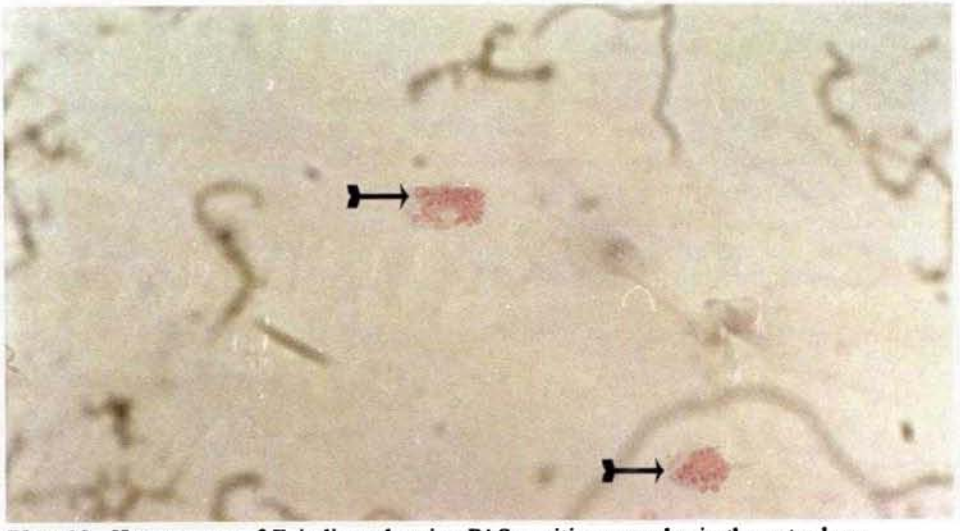

Plate 13. Haemocytes of *F. indicus* showing PAS positive granules in the cytoplasm, indicating the presence of carbohydrates. Note the pink coloured granules (arrow) in the cytoplasm. 1000X.

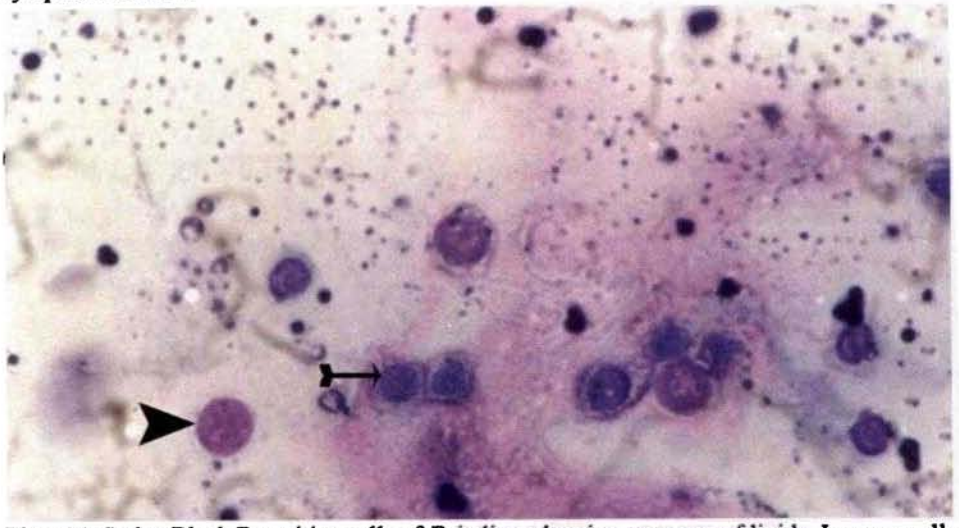

Plate 14. Sudan Black B positive cells of *F. indicus* showing presence of lipids. In some cells (arrow) dark areas are seen towards cell periphery, while other cells (arrow head) contain dark granules. 1000X.

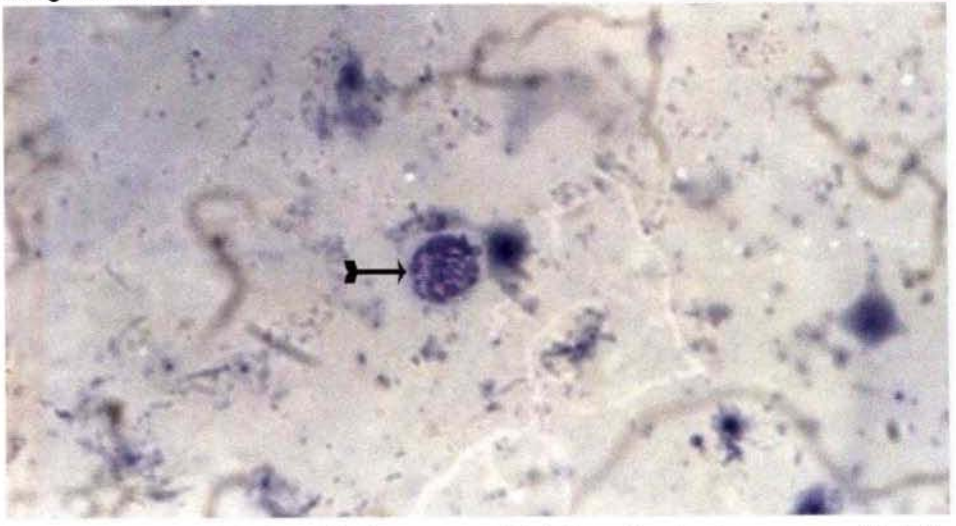

Plate 15. A Sudan Black B positive haemocyte of *F. indicus* showing the presence of lipid in the cytoplasm. Note the distinct dark granules (arrow). 1000X

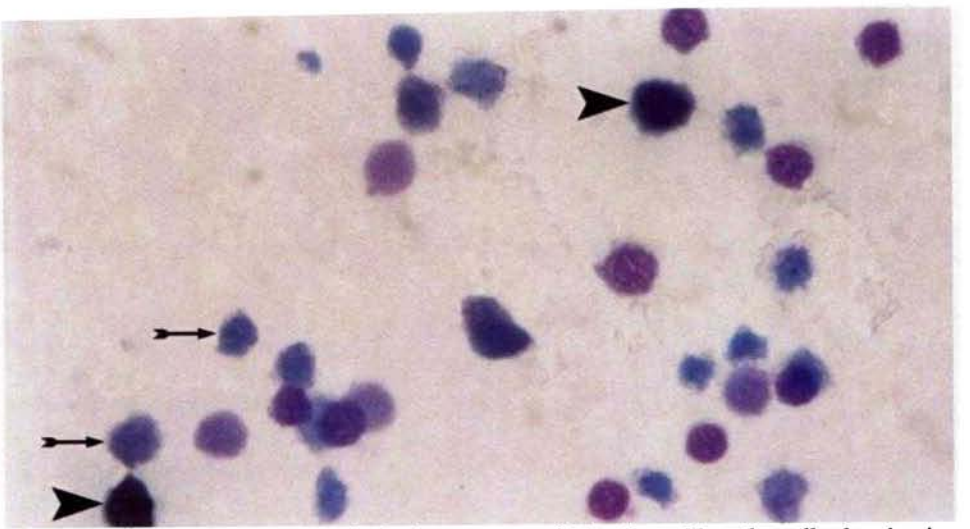

Plate 16. Phenoloxidase activity in the haemocytes of *F. indicus*. Note the cells showing intense activity (arrow head). Cells showing moderate activity are also seen (arrow). 1000X.

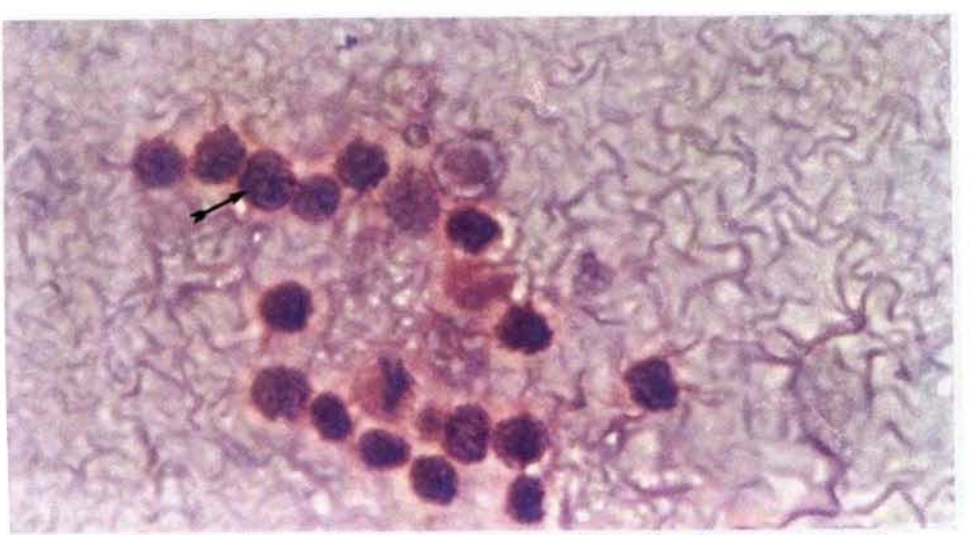

Plate 17. Myleoperoxidase reaction in the haemocytes of *F. indicus* displaying peroxidase activity. Note the dark staining granules. 1000X.

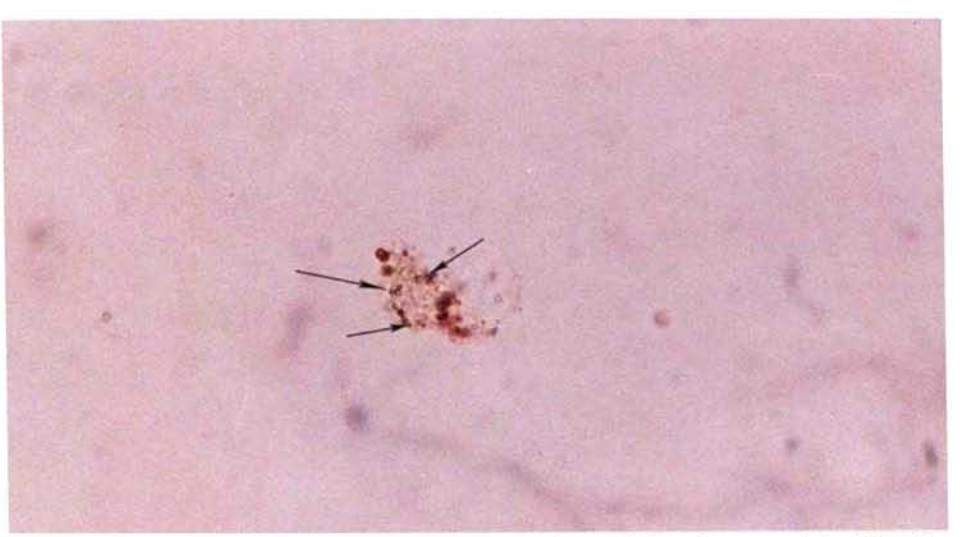

Plate 18. Cytoplasmic granules of a haemocyte exhibiting acid phosphatase activity. Note the blue granules .1000X.

Table 1. Cellular factors of apparently normal *Fenneropenaeus indicus*

Sample. No.	THC ($\times 10^4$cells/ml)	DHC (%)			% phagocytosis
		Hy	SGH	LGH	
1	1647	28	67	5	67
2	1396	26	69	6	59
3	1315	21	77	3	63
4	1907	24	71	5	55
5	2333	20	69	5	39
6	2205	21	71	4	49
7	2264	23	54	10	50
8	2431	24	69	11	50
9	2311	20	63	9	45
Mean	**1978.77**	**22.92**	**67.77**	**6.44**	**53.15**
±SD	**4.28**	**2.78**	**6.35**	**2.8**	**8.87**

THC = Total haemocyte count

DHC = Differential haemocyte count as percentage of different haemocytes

Hy = Hyalinocytes

SGH = Small-granule haemocytes

LGH = Large-granule haemocytes

% Phagocytosis= number of haemocyts phagocytising yeast cells in 100 haemocytes

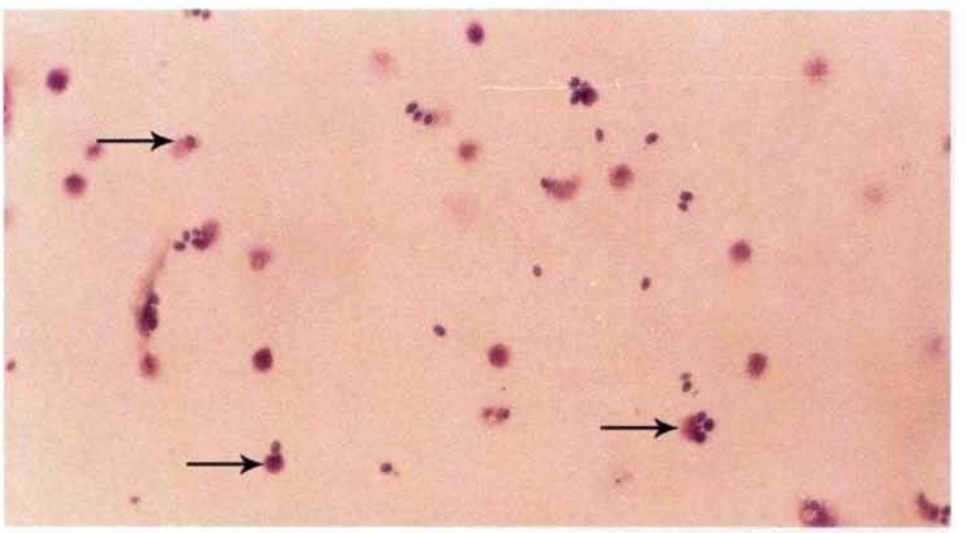

Plate 19. Phagocytic activity (arrow) of the haemocytes of *F. indicus*. Wright's - Giemsa stain. 400X.

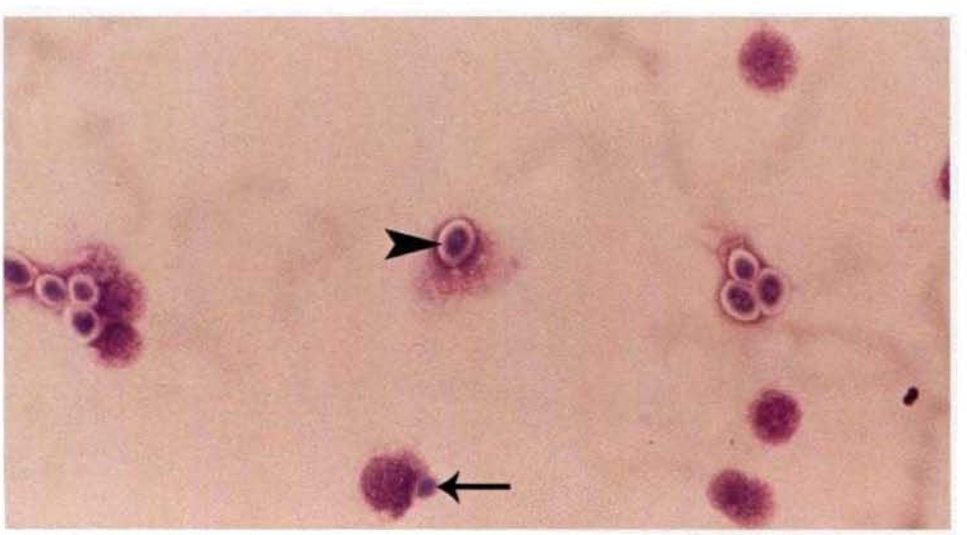

Plate 20. Haemocytes of *F. indicus* showing phagocytosis of yeast cells. Note the attached (arrow) and engulfed (arrow head) yeast cell. Wright's Giemsa stain. 1000X.

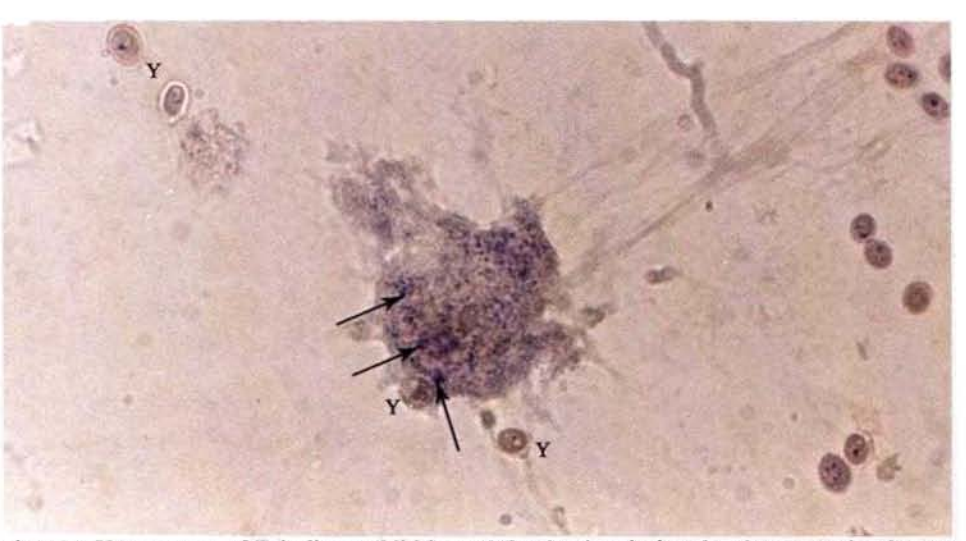

Plate 21. Haemocytes of *F. indicus* exhibiting NBT reduction during the phagocytosis of yeast cells (Y). Note the dark blue formazan granules, indicating oxygen radical production. 1000X.

4.2.2. Humoral factors

4.2.2.1. Protein profile

4.2.2.1.1. Total serum protein

The total serum protein concentrations of apparently healthy animals ranged between 57 mg/ml and 72 mg/ml, with a mean value of 61.16±1.92 mg/ml (Table 2).

4.2.2.1.2. SDS-PAGE of serum

The SDS-PAGE profile of the serum by SDS-PAGE revealed 14 - 16 polypeptide fractions. Among these, one fraction was of high intensity. The high intensity polypeptide fraction was of molecular weight between 70 and 80 kDa. The other fractions were of moderate intensity and displayed a molecular weight of 56, 44, 41, 40, 38, 24 and 20 kDa. The rest of the bands were feeble. Of these, two to three bands weighed more than 98 kDa. The other low intensity fractions were of molecular weight between 54 and 27 kDa (Plate 22).

4.2.2.2. Haemagglutination

The serum of apparently healthy animals was able to agglutinate chicken red blood cell suspension. There was no significant change in titer value when incubated at room temperature for 1 hour and at 4°C for 24 hours. The titer, expressed as the reciprocal of the maximum dilution giving positive agglutination, varied from 16 to 64. The mean value was 33.77 ± 18.66 (Table 2).

4.2.2.3. Enzyme assays

4.2.2.3.1. Phenol oxidase

Phenol oxidase activity in the haemolymph of apparently healthy shrimp is given in Table 2. The haemolymph showed phenoloxidase activity ranging from 0.667 to 2.57 enzyme units, with a mean value of 1.1640 ± 0.59 enzyme units.

4.2.2.3.2. Serum acid phosphatase

The concentration of acid phosphatase in the serum of apparently healthy animals was in the range of 26.12 to 42.9 KA units and the mean value was 33.59 ± 7.5 KA units (Table 2).

4.2.2.3.3. Serum alkaline phosphatase

Serum alkaline phosphatase activity of apparently normal *F. indicus* is given in Table 2. The mean serum alkaline phosphatase activity recorded was 2.309 ± 0.318 KA units, and ranged between 2.055 and 3.04 KA units.

4.3. Histology of tissues of apparently normal *F. indicus*

4.3. 1. Gills

Histological studies showed that the gills branch from a central axis into primary filaments. Each primary filament divides into secondary filaments which are branching and non-branching. Each secondary filament consists of epithelial pillar cells. Interspaced between the pillar cells are interconnecting spaces or lacunae, the tips of which are enlarged. Afferent and efferent vessel carrying haemolymph is observed in each secondary filament. Endothelial cells line both the vessels (Plate 23).

4.3.2. Heart

The heart is situated dorso-posterior to the hepatopancreas and is surrounded by the pericardium or the pericardial chamber. Immediately surrounding the heart is the epicardium composed of specialized spongy connective tissue. The heart is composed of myocardial cells arranged in bands, which are primarily organized into distinct bundles. Satellite cells are seen in between the myocardial cells. The pericardium is surrounded by the pericardial septum, which is thick and spongy dorsally and laterally, while thin and dense ventral to the heart. The lumenal sub-chambers are in the center of the heart proper. The posterior and anterior aorta are also observed (Plate 24).

4.3. 1.3. Haematopoietic tissue

Haematopoietic tissue of *F. indicus* is located around the lateral arterial vessel and at the base of the maxillipeds. It is also present laterally to the anterior stomach chamber and is seen interspaced between the antennal tubules. The region anterior to the stomach and posterior to the brain also contains haematopoietic tissue. The tissue is arranged into distinct lobules, with each lobule limited by thin fibrous connective tissue. Young developing haemocytes and haemal sinuses are interspaced between the lobules, where mitotic figures are frequently seen (Plate 25).

4.3. 1.4. Hepatopancreas

The tissue is organized into hepatic tubules, which are divided into undifferentiated apical region, differentiating medial region, and differentiated proximal region. The lumen of the tubule contains a granular matrix and is in continuation with the secondary duct. The apical region consists of undifferentiated E-cells. The medial region is made of two types of cells. The first type is the R-cells, which are developing, absorptive, storage cells. Their cytoplasm contains numerous vacuoles. The second type is the F-cell. These cells are more basophilic when further away from the apex. Their nuclei are larger than the R-cells and contain nucleoli. In addition to R-cells and F-cells the proximal region contains B-cells, which have a single prominent vacuole, and a convex luminal surface. The R-cells, F-cells, and B-cells have a brush-border surface that faces the lumen. Myoepithelial cells and contractile fibres form a network around the tubule. The intertubular space is comprised of haemal sinus (Plate 26).

4.3. 1.5. Antennal gland

The antennal gland tubules are composed of simple columnar epithelial cells. The cells have a medial nucleus with a well-defined nucleolus. The surface of the cells is brush-like. Surrounding the antennal tubules are haemolymph sinuses that contain haemocytes (Plate 27).

Table 2. Humoral factors of apparently normal *F. indicus*

Sample. No.	TSP (mg/ml)	HA (1/titer)	PO (enzyme units)	SAcP (KA units)	SAlP (KA units)
1	67	32	1.11	22.9	3.04
2	72	64	2.57	26.12	2.55
3	67.5	64	1.53	27.42	2.16
4	57.5	32	0.75	41.9	2.06
5	57.5	32	0.67	41.1	2.15
6	56	32	0.71	42.9	2.11
7	45	16	1.11	37.9	2.34
8	43	16	0.97	32	2.06
9	34.5	16	1.06	30.1	2.34
Mean	**55.55**	**33.77**	**1.16**	**33.59**	**2.31**
±SD	**12.57**	**18.66**	**0.59**	**7.52**	**0.31**

TSP = Total serum protein

HA = Haemagglutination (Reciprocal of titer giving maximum dilution)

PO = Phenoloxidase (enzyme units=Δ 0.001 O.D/min /mg of protein)

SAcP= Serum acid phosphatase

SAlP= Serum alkaline phosphatase

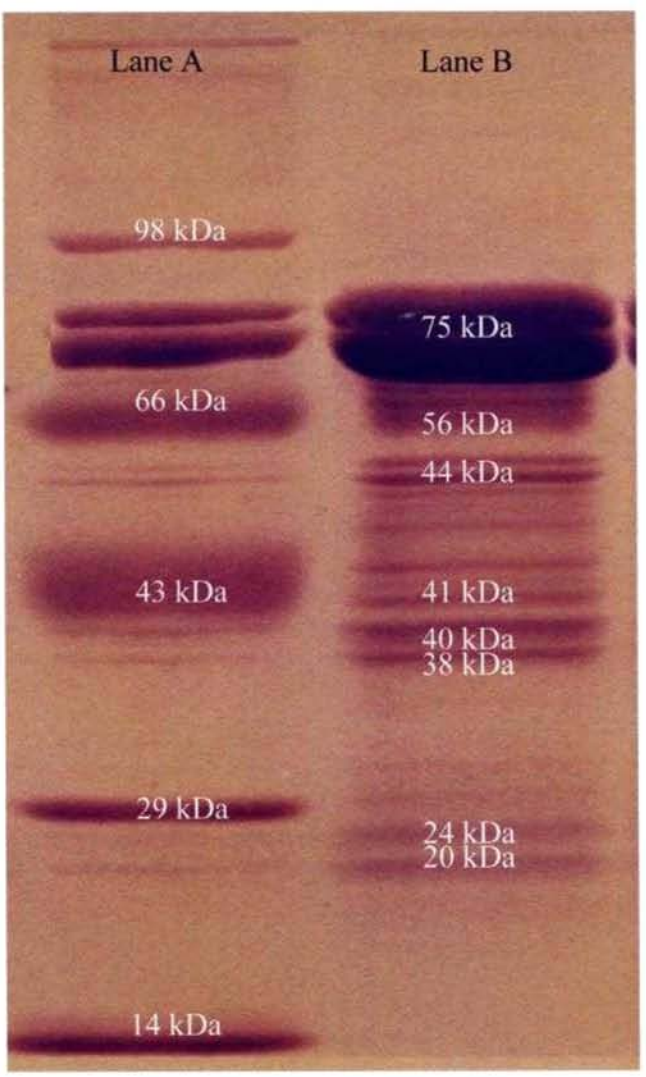

Plate 22. SDS-PAGE of serum of apparently normal *F. indicus*. Lane A- Molecular marker; Lane B- serum of *F. indicus*

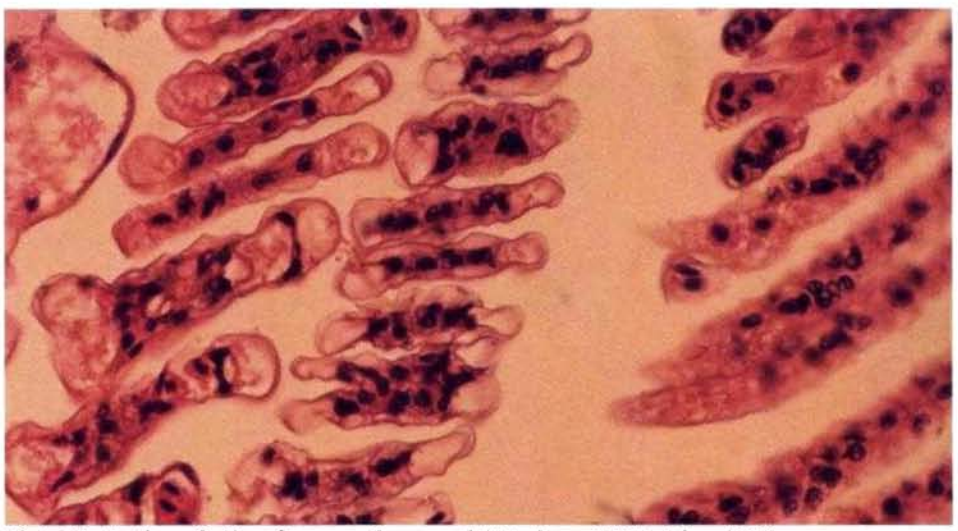

Plate 23. Section of gills of apparently normal *F. indicus*. H&E stain. 400X.

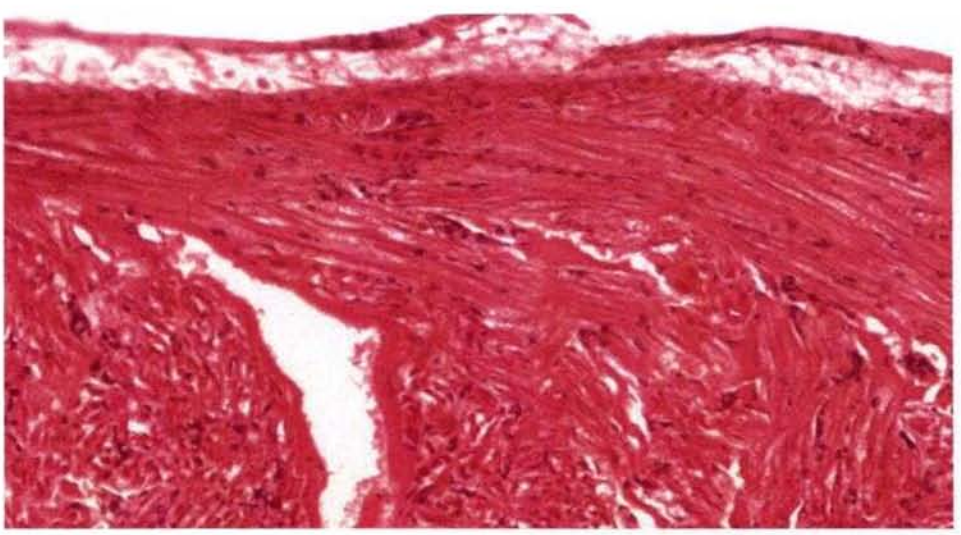

Plate 24. Section of heart of apparently normal *F. indicus*. H&E stain. 400X.

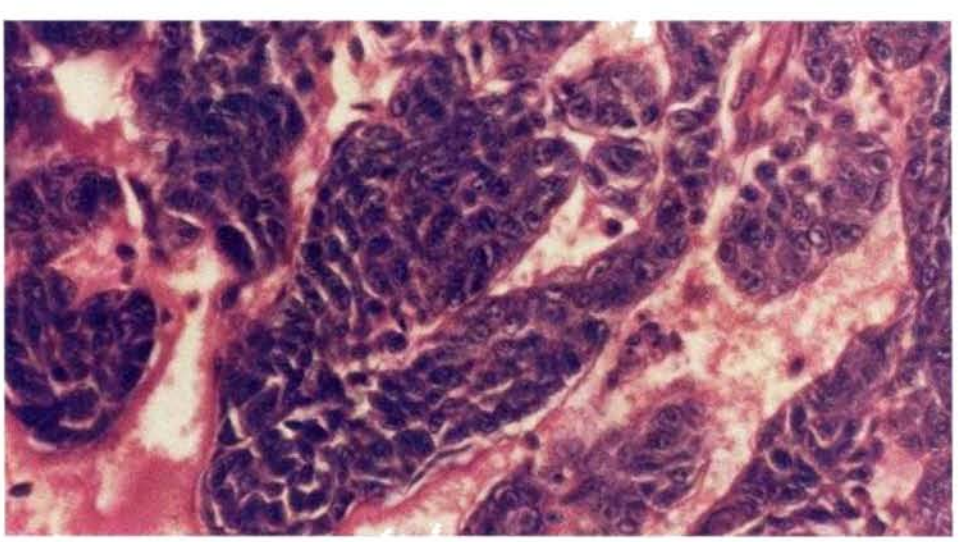

Plate 25. Section of haematopoietic tissue of apparently normal *F. inducus*. H&E stain. 400X.

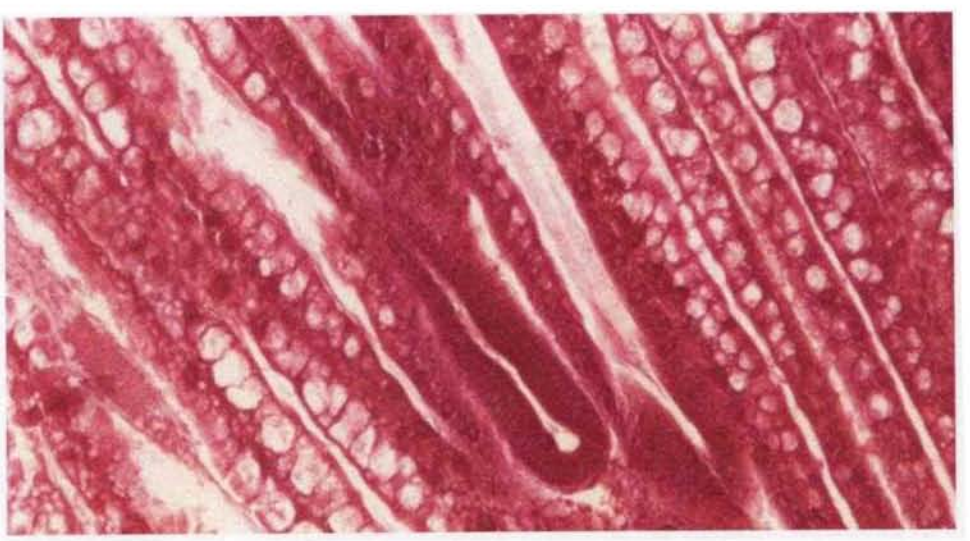

Plate 26. Section of hepatopancreas of apparentely normal *F. indicus*. H&E stain. 400X.

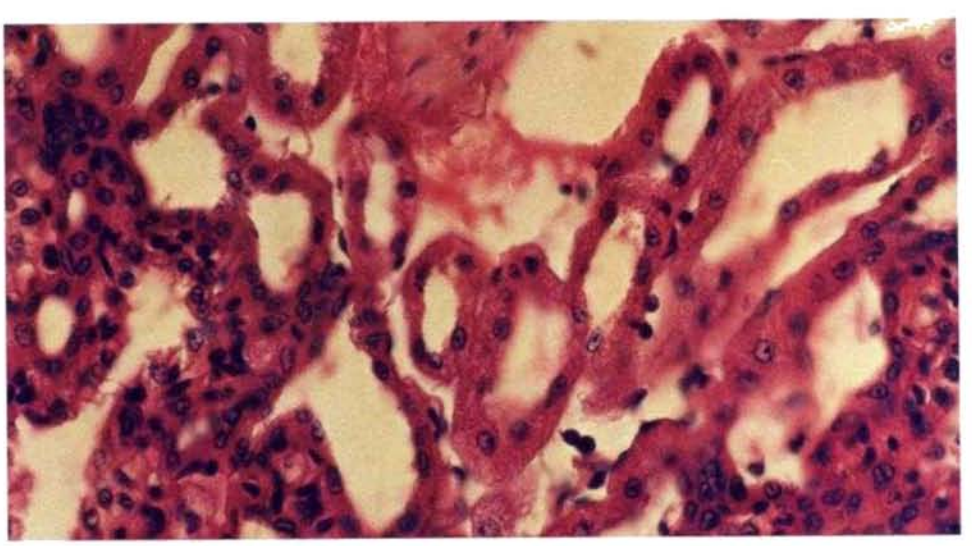

Plate 27. Section of normal antennal gland of apparently normal *F. indicus*. H&E stain. 400X.

4.4. Immunomodulation

4.4.1. Exposure of *F. indicus* to sub-lethal dose of *V. parahaemolyticus*

4.4.1. 1.Cellular factors

4.4.1.1.1. Total haemocyte count (THC)

The animals of *F. indicus* were exposed to a sub-lethal dose of the pathogen and the recorded total haemocyte count during 30 min, 3 h, 24 h, 48 h, 72 h, 1 week and 2 weeks are given in the Table 3. At 30 min and 24 h of injection, the number of circulating cells in the test animals was significantly lower ($p < 0.05$) than that of the control group. But at 72 h, the total count of the test animals displayed a significant increase ($p< 0.05$) as compared to the control animals. After one week of injection, the total haemocyte counts of the control and test animals were comparable (Fig. 1).

4.4.1.1.2. Differential heamocyte count (DHC)

The differential haemocyte count of the control and bacteria injected animals are given in Table 3.

There was an initial elevation in the mean percentage hyalinocytes of the test animals as compared with the control animals, which was significantly ($p< 0.05$) so at 48 h post injection. But after 48 h, their percentage in the circulation in the test animals decreased, and was lower than the control values (Fig. 2).

In general, the recorded percentage of small granulae haemocytes of the test animals was lower than the control upto 48 h. However only at 24 h the drop was significant ($p < 0.05$). Afterwards, the percentage of these haemocytes showed an increase and was greater than the control (Fig. 3).

Although the percentage of large granule haemocytes was greater than the control at 30 min, their percentage decreased. The level of the large granule haemocytes in the test was significantly lower than the control ($p < 0.05$) at 3 h (Fig. 4).

4.4.1.1.3. Phagocytic activity

There was no significant difference ($p > 0.05$) in the *in vitro* phagocytic activity using yeast cells in *F. indicus* (Table 5 and Fig. 5).

Table 3. Total haemocyte count (× 10^4 cells/ ml of haemolymph) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ml) at different time intervals

Treatments \ Time interval		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean ±SD	2401.66 ± 298.58	1440.66 ± 610.64	3282.66 ± 432.46	1938.33 ± 104.50	1814.0 ± 53.32	2218.66 ± 268.77	2335.33 ± 86.11
Test	Mean ±SD	668.33* ± 99.57	1220.33 ± 176.13	361.66* ± 65.60	1109.00* ± 192.03	3162.33* ± 541.12	2502.0± 364.08	2441.33± 583.80

Values with * differ significantly from the control (p< 0.05)

Table 4. Differential haemocyte count (percentage of cells) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ ml), during different time intervals

Differential haemocyte count (%)[Mean±SD] \ Time intervals		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Hyalinocytes	Control	16.0±3.0	15.66±3.05	11.0±3.0	10.66±2.08	17.0±5.29	12.66±1.52	10.33±4.5
	Test	18.0±2.0	16.0±3.46	14.66±1.52	15.66*±2.88	15.0±3.46	12.66±0.57	8.33±5.03
Small granule haemocytes	Control	73.33±2.51	72.0±4.35	80.0±2.0	80.0±3.0	75.0±5.56	74.66±3.05	82.66±7.02
	Test	67.0±7.0	76.0±3.46	73.0*±2.64	72.66±3.78	81.33±3.05	78.33±1.52	85.0±7.0
Large granule haemocytes	Control	10.66±0.57	12.33±2.51	9.0±1.0	9.33±1.55	8.0±2.64	12.66±4.04	7.0± 2.64
	Test	15.33±4.61	8.0*±0.0	11.66±3.51	11.66±3.05	3.66±1.15	8.66±1.52	6.66±3.78

Values with * differ significantly from the control (p< 0.05)

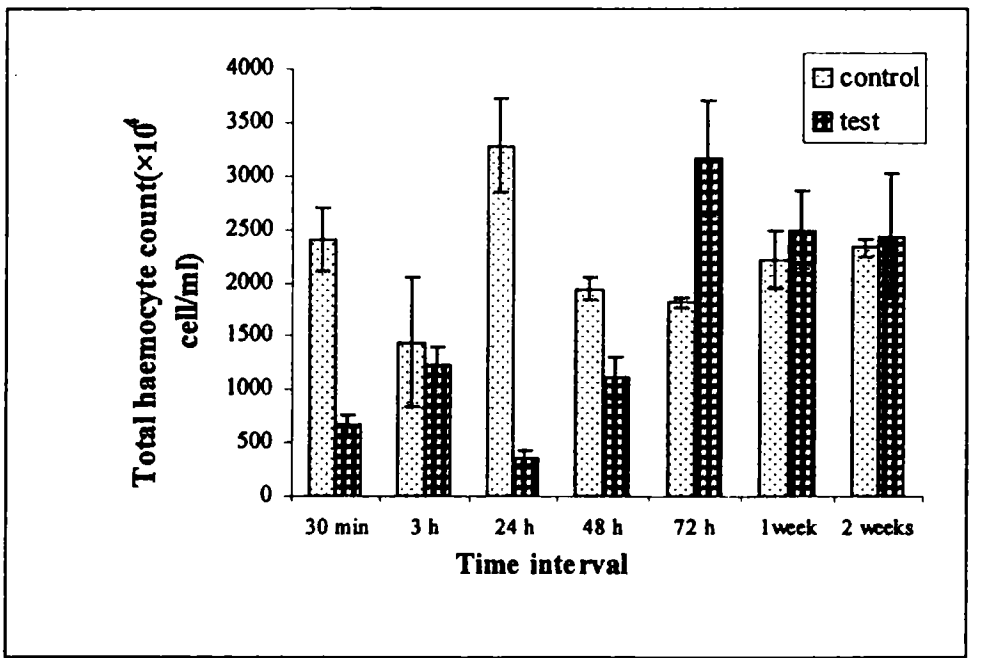

Fig .1. Total haemocyte count (× 10^4 cells/ml haemolymph) of *F. indicus* exposed to sub-lethal dose of *V. parahaemolyticus* at different time intervals

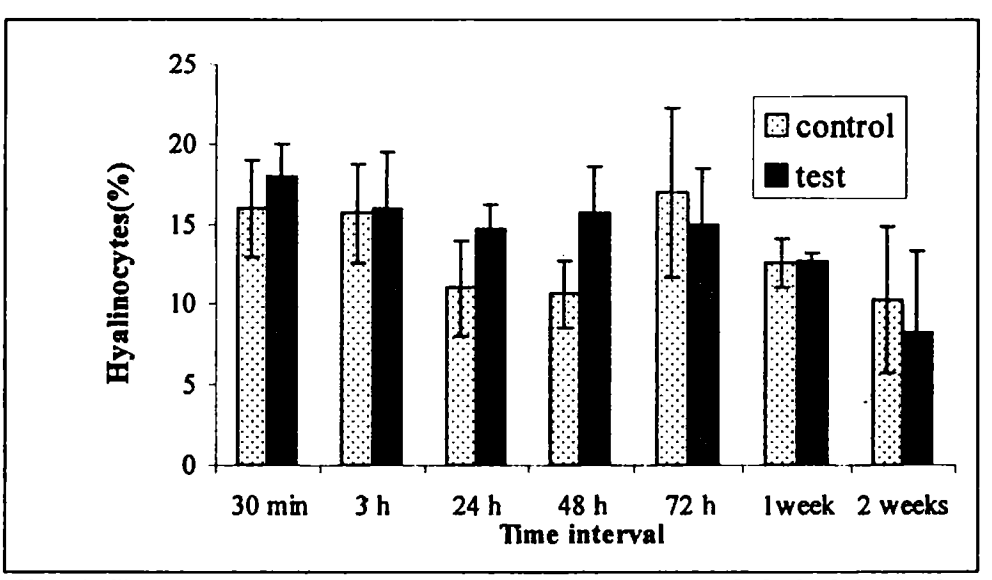

Fig. 2. Hyalinocytes (%) of *F. indicus* exposed to a sub-lethal dose of *V. parahaemolyticus* at different time intervals

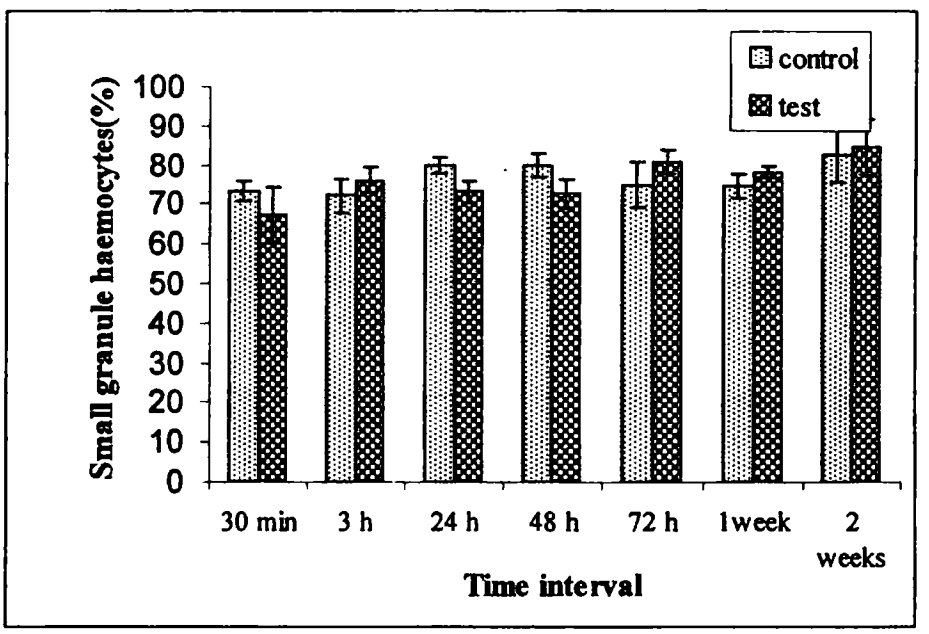

Fig.3. Percentage of small granule haemocytes of *F. indicus* exposed to sub-lethal dose of *V. parahaemolyticus* at different time intervals

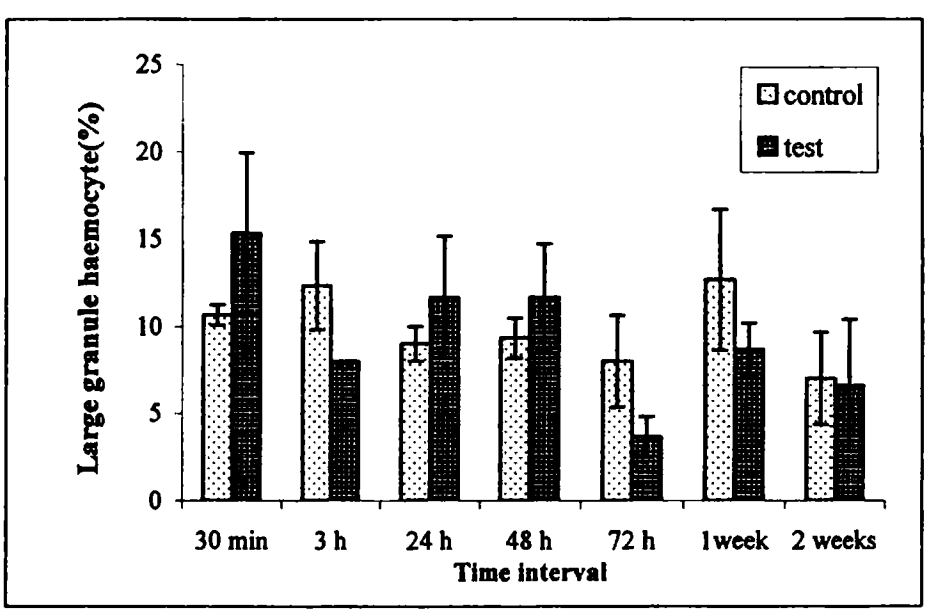

Fig. 4. Percentage of large granule haemocytes of *F. indicus* exposed to a sub-lethal dose of *V. parahaemolyticus* at different time intervals

Table 5. Phagocytic activity (% phagocytosis) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ ml) at different time intervals

Time interval / %Phagocytosis		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean± SD	58.66±4.16	56.33±2.05	61.33±2.3	60.33±1.52	40.33±7.23	55.66±4.5	42.0±5.0
Test	Mean ±SD	60.0±1.0	57.0±2.0	67.0±3.0	59.66±1.52	41.0±2.64	52.0±3.0	36.0±3.46

There was no significant difference between treatments ($p > 0.05$)

Table 6. Total serum protein concentration (mg/ ml) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ ml) at different time intervals

Time interval / TSP(mg/ml)		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean ±SD	43.66± 1.15	36.0± 0.0	37.5± 0.86	44.83± 1.44	45.83± 1.04	37.33± 1.6	28.83± 0.57
Test	Mean ±SD	34.16*± 0.57	42.33*± 3.79	48.0*± 1.0	41.17*± 0.76	39.5*± 1.32	28.83*± 1.15	30.33± 0.76

Values with * differ significantly from the control ($p< 0.05$)

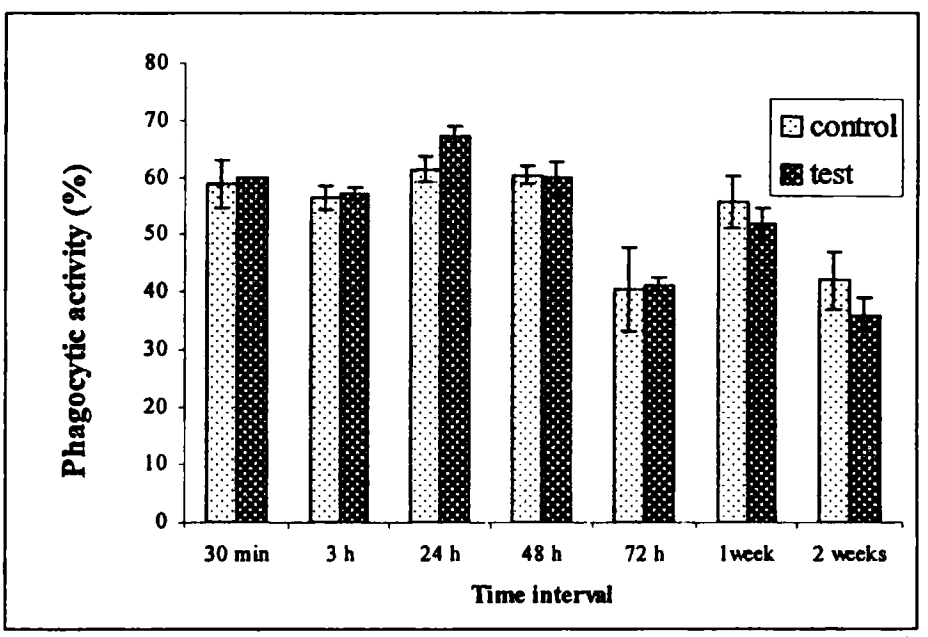

Fig. 5. Phagocytic activity (%) of haemocytes of *F.indicus* exposed to sub-lethal dose of *V. parahaemolyticus*

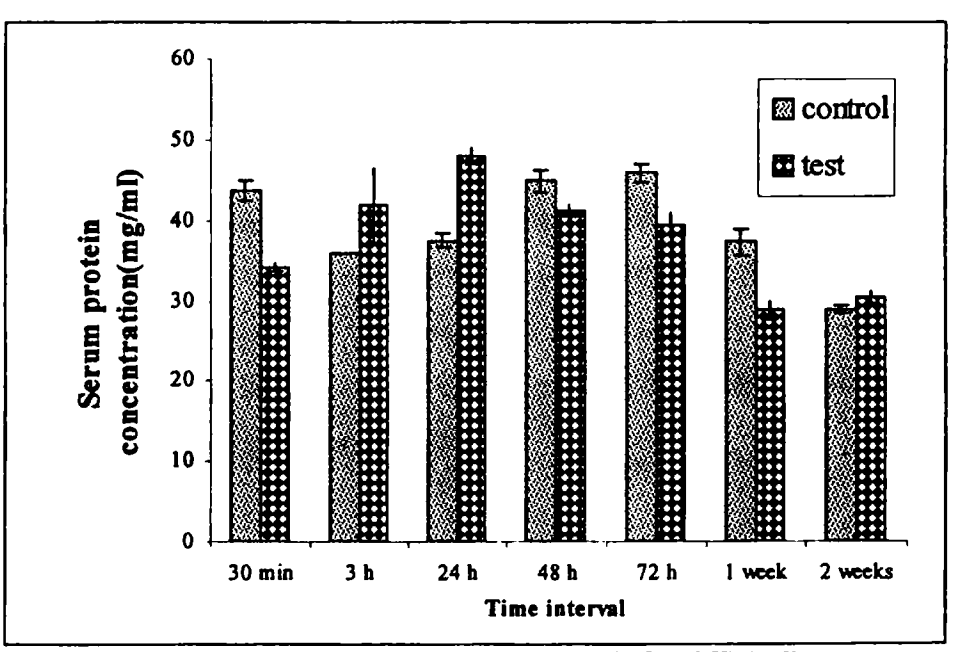

Fig. 6. Total serum protein concentration (mg/ml) of *F. indicus* exposed to sub-lethal dose of *V. parahaemolyticus*

4.4.1.2. Humoral factors

4.4.1.2.1. Total serum protein

The mean total protein concentration of the test animals was significantly lower ($p < 0.05$) than the control at 30 min post injection (Table 6). But at 3 h and 24 h, there was a significant increase ($p < 0.05$) in the protein concentration of the test animals than the control. After 24 h, the level of serum protein in the bacteria injected animals decreased significantly ($p < 0.05$) (Fig. 6).

4.4.1.2.2. SDS-PAGE

There was no observable variation in the SDS-PAGE of the serum of the animals of control and test groups.

4.4.1.2.3. Haemagglutination

The haemagglutinating activity of the control and test group is given in Table 7. The haemagglutinating activity of the test and control showed the same increase in titer till 24 h (Fig. 7). After this the haemagglutinating activity was significantly greater ($p < 0.05$) than the control till 72 h, which decreased afterwards

4.4.1.2.4. Enzyme assays

4.4.1.2.4.1. Phenoloxidase (PO)

Phenoloxidase activity of the experimental animals is given in Table 8. The phenoloxidase activity of the test animals was significantly lower ($p < 0.05$) than the control at 30 min. Afterwards, a rise was seen in the enzyme activity in the test animals, which was significantly ($p < 0.05$) higher than the control animals at 3h, 1 week and 2 weeks of post injection (Fig. 8).

4.4.1.2.4.2. Serum acid phosphatase

Initially, the mean serum acid phosphatase activities of the test animals was significantly lower ($p <0.05$) in the test animals than the control up to 3 h (Table 9). However, at 24 h and 2 weeks of post injection, the acid phosphatase activity was significantly higher (p 0.05) than the enzyme activity of the control animals (Fig. 9).

4.4.1.2.4.3. Serum alkaline phosphatase

Table 10 shows the mean serum alkaline phosphatase concentration of the two groups of animals during the different intervals. The alkaline phosphatase activity of the test animals was significantly lower ($p < 0.05$) than the control after bacterial injection (Fig. 10).

4.4.1.3. Histology

At 3 hours of injection, engorgement of sinuses of gill filaments with haemolymph, and exudation was observed (Plate 28). The chambers of the heart were engorged with haemolymph with some of the myocardial bundles showing loss of striation (Plate 29). The haemopoietic tissues appeared normal with haemocytes in the dividing stages and sinuses containing haemocytes. In the hepatopancreas, there were a large number of vacuolated R-cells, with few numbers of F-cells. B-cells were few in the proximal region. The haemolymph sinuses between the tubules were enlarged and engorged with haemolymph (Plate 30). Exudation of proteinaceous substances was evident between the antennal gland tubules. Along with this, moderate infiltration of haemocytes between the tubules of the labyrinth was observed (Plate 31).

After 24 h, the haemolymph sinuses of the gills displayed engorgement (Plate 32), which remained so till 48 h. Mild exudation was seen between the tubules of the antennal gland after 48 h. The other tissues did not display any discernable variation.

Table 7. Haemagglutinating activity (reciprocal of titer value) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ ml) at different time intervals

Time interval / Haemagglutination		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean ±SD	64±0	64±0	256±0	128±0	128±0	128±0	64±0
Test	Mean ±SD	64±0	64±0	256±0	256*±0	256*±0	128±0	64±0

Values with * differ significantly from the control ($p< 0.05$)

Table 8. Phenoloxidase activity (enzyme units) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ ml) at different time intervals

Time interval / PO(enzyme units)		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean ±SD	0.454± 0.039	0.248± 0.02	0.623± 0.212	1.012± 0.14	0.796± 0.092	1.050± 0.049	0.66± 0.033
Test	Mean ±SD	0.368*± 0.013	0.382*± 0.012	1.009± 0.161	1.014± 0.174	0.705± 0.053	1.815*± 0.139	1.777*± 0.128

Values with * differ significantly from the control ($p< 0.05$)

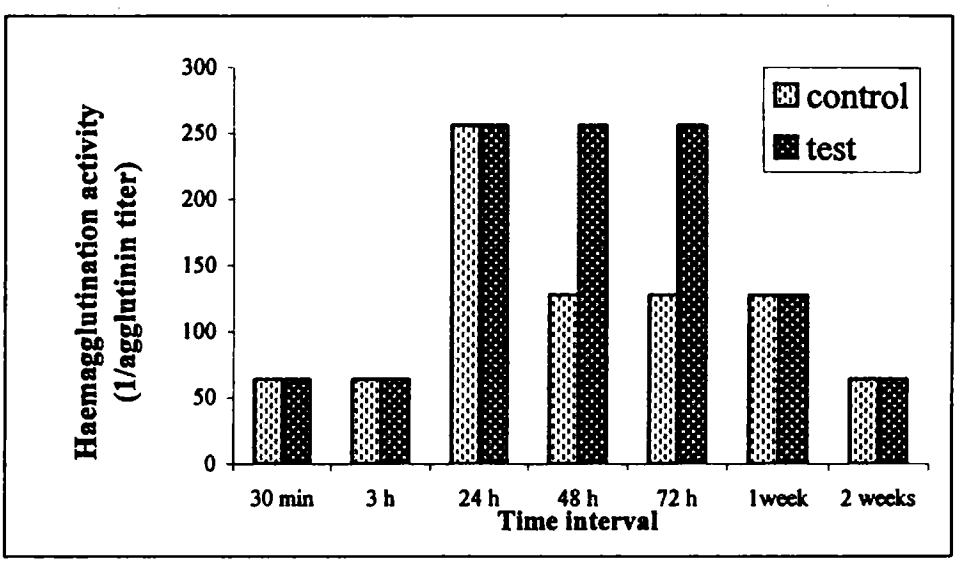

Fig. 7. Haemagglutination titer (reciprocal of dilution giving maximum agglutination of chicken red blood cells) of *F. indicus* exposed to *V. parahaemolyticus*

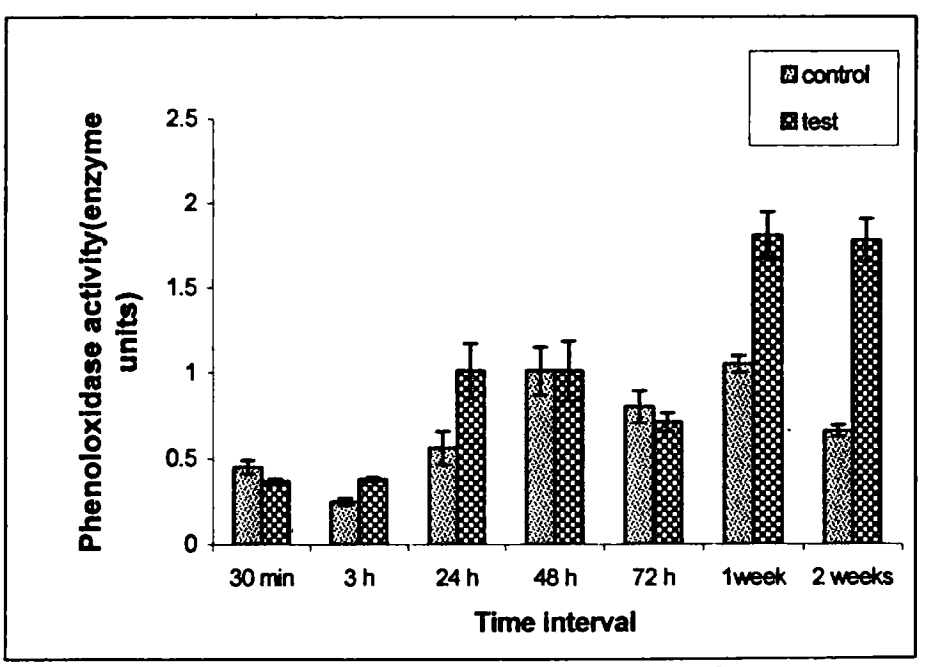

Fig. 8. Phenoloxidase enzyme activity (enzyme units) of *F. indicus* exposed to sub-lethal dose of *V. parahaemolyticus*

Table 9: Serum acid phosphatase levels (KA units) of *F. indicus* exposed to *V. parahaemolyticus* (10^4cells/ ml) at different time intervals

Time interval / SAcP(KA units)		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean ±SD	33.33± 4.06	55.03± 8.32	22.33± 0.57	47.0± 2.64	38.66± 2.51	28.0± 2.64	22.0± 1.0
Test	Mean ±SD	33.0± 0.86	38.7*± 3.45	26.66*± 0.57	35.66*± 0.57	39.66± 3.51	21.0*± 1.0	30.3*± 1.73

Values with * differ significantly from the control ($p< 0.05$)

Table 10. Serum alkaline phosphatase levels (KA units) of *F. indicus* exposed to *V. parahaemolyticus* (10^4 cells/ ml) at different time intervals

Time interval / SAIP(KA units)		30 min	3 h	24 h	48 h	72 h	1week	2 weeks
Control	Mean ±SD	2.58±0.44	2.15±0.09	3.04±0.17	3.82±0.8	2.06±0.1	1.67±0.14	1.48±0.19
Test	Mean ±SD	1.4*±0.11	1.4*±0.05	0.95*±0.15	2.65*±0.1	1.95±0.09	1.13*±0.25	1.97±0.37

Values with * differ significantly from the control ($p< 0.05$)

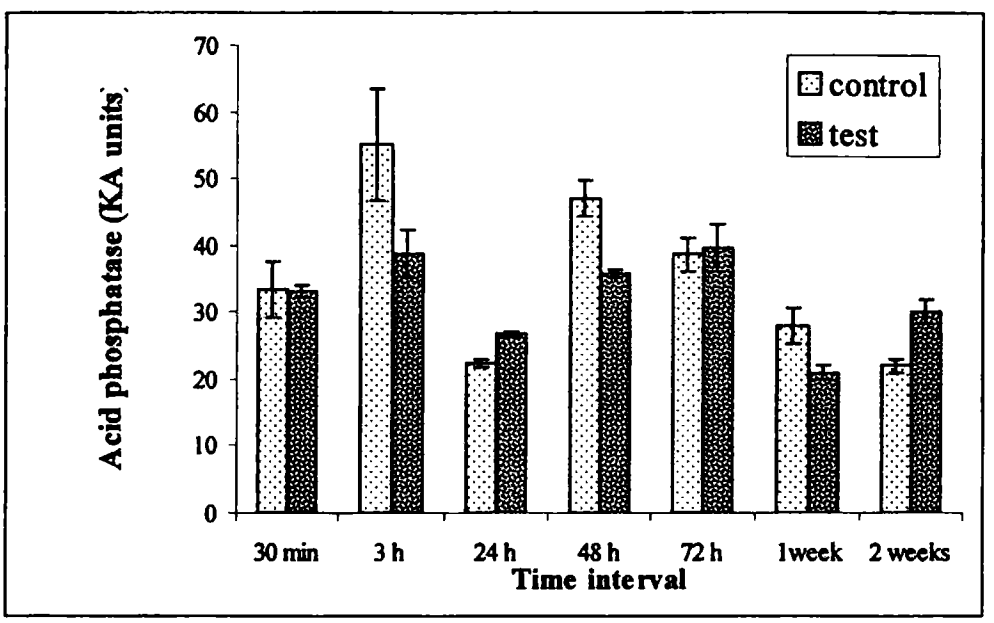

Fig. 9. Concentration of serum acid phosphatase enzyme (KA units)of *F. indicus* exposed to sub-lethal concentration of *V.parahaemolyticus*

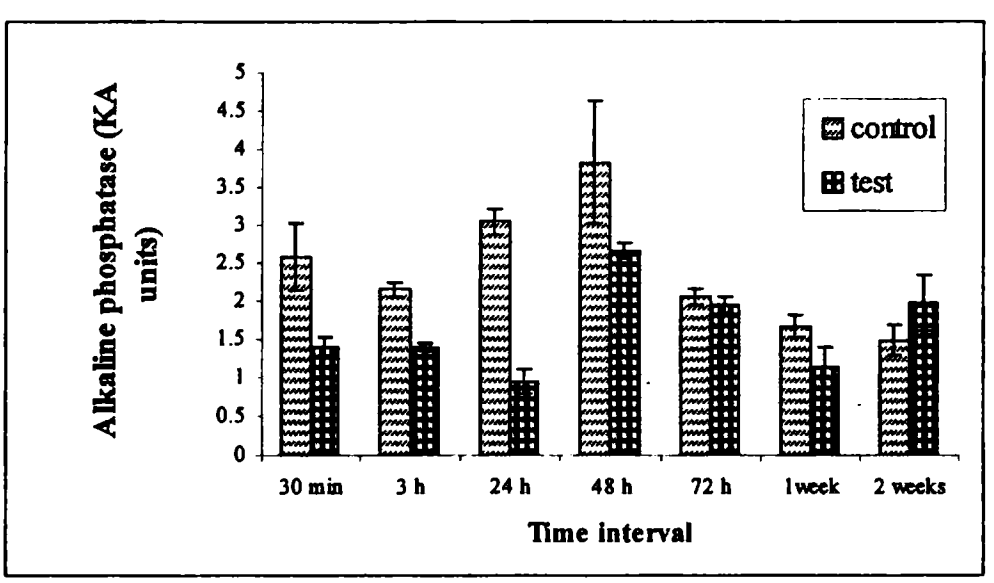

Fig.10. Concentration of serum alkaline phosphates enzyme (KA units)of *F. indicus* exposed to sub-lethal concentration of *V. parahaemolyticus*

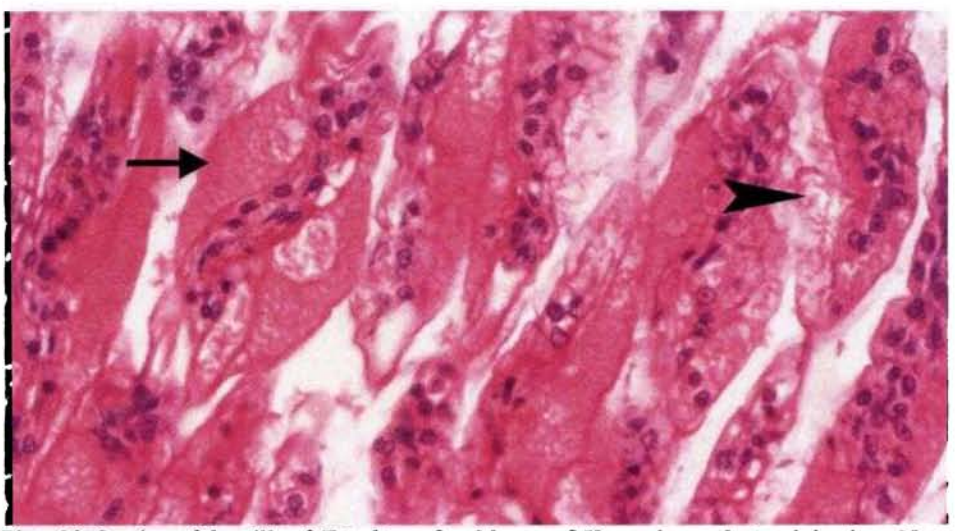

Plate 28. Section of the gills of *F. indicus* after 3 hours of *V. parahaemolyticus* injection. Note the engorgement of haemal sinuses with coagulated haemolymph (arrow) and also exudation of haemolymph into inter-filamental region (arrow head). H&E stain. 400X.

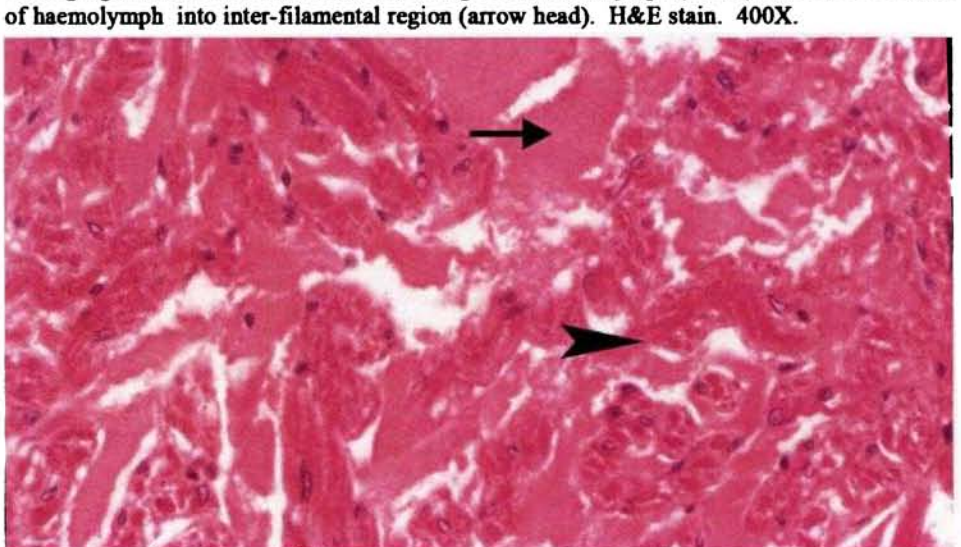

Plate 29. Section of heart of *F. indicus* after 3 hours of *V. parahaemolyticus* injection. Note the engorgement of spaces with haemolymph (arrow) and loss of striation in the muscle fibres (arrow head). H&E stain. 400X.

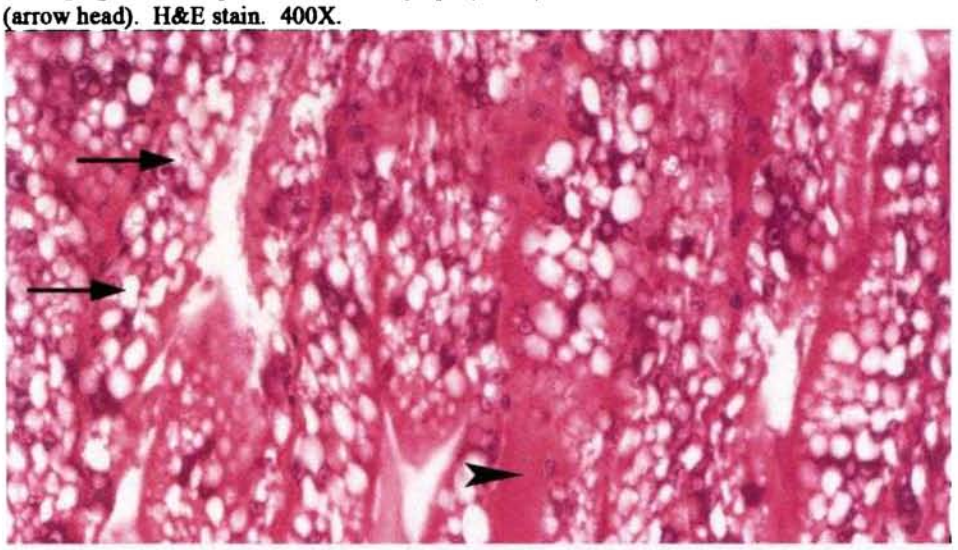

Plate 30. Section of hepatopancreas of *F. indicus* after 3 hours of *V. parahaemolyticus* injection showing abundant R cells (arrow) and aslo the engorgement of haemal sinuses (arrow head). H&E stain. 400X.

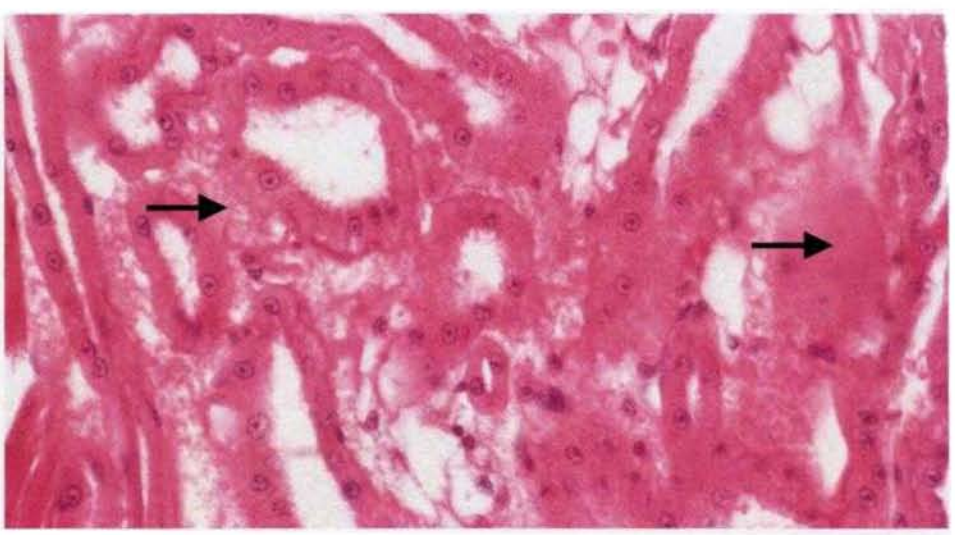

Plate 31. Section of antennal gland of *F. indicus* after 3 hours of *V. parahaemolyticus* injection. Note the severe exudation between tubules of the antennal gland (arrow). H&E stain. 400X.

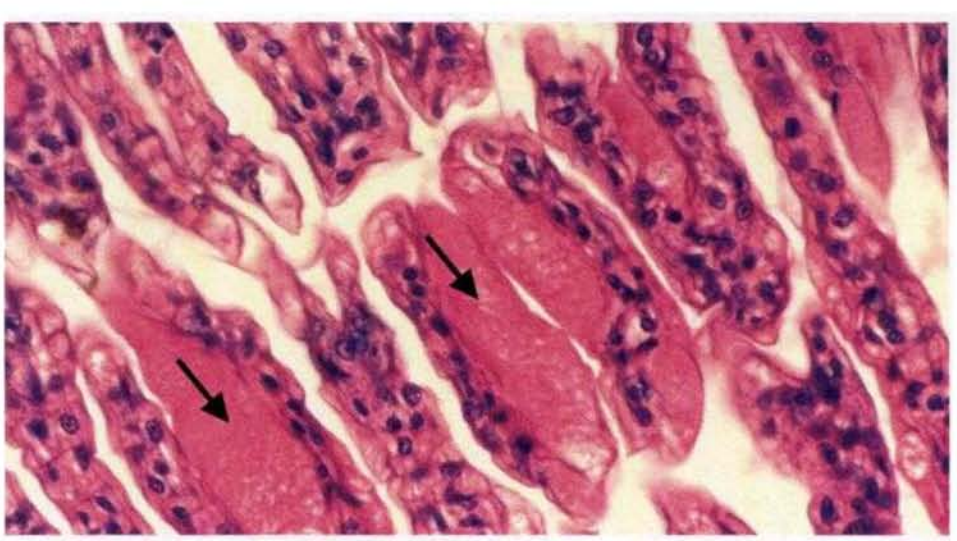

Plate 32. Section of gill of *F. indicus* after 24 hours of *V. parahaemolyticus* injection. Note the engorgement of haemal sinuses of secondary gill filaments (arrow). H&E stain. 400X.

4.4.2. Effect of exposure to nuvan

4.4.2.1. Cellular factors

4.4.2.1.1. Total haemocyte count (THC)

On exposure to different concentrations of nuvan, the mean total haemocyte count of the animals demonstrated a general increase, with exception to animals exposed to 0.02 ppm concentration (Table 11). In animals exposed to 0.02 ppm nuvan, a significant decrease ($p<0.05$) in the total count was noticed. However, in shrimp exposed to 0.05 and 0.08 ppm nuvan, there was an increase in mean total haemocyte counts. The mean THC of 0.05 ppm exposed group displayed a value of $2225.66 \pm 48.05 \times 10^4$ cells/ml. In animals reared in waters of 0.08 ppm nuvan, a mean THC value of 3007.33 ± 196.87 10^4cells /ml was observed, which was significantly greater ($p < 0.05$) than the control value (Fig.11).

4.4.2.1.2. Differential haemocyte count

The mean differential haemocyte count of the control and nuvan treated animals are given in the Table 11 and Fig.12. The mean percentage hyalinocytes of control animals exhibited a value of $21.0 \pm 1.0\%$. The animals exposed to 0.05 ppm nuvan displayed a significant rise ($p< 0.05$) in hyalinocyte count. In the animals exposed to 0.02 and 0.08 ppm nuvan percentage of hyalinocytes was similar to the value of control animals. A significant decrease ($p<0.05$) in the percentage of small granule haemocytes was noticed in the 0.05 ppm nuvan exposed animals. The animals exposed to 0.02 and 0.08 ppm concentrations of nuvan showed an increase in percentage of small granule haemocyte, but was not statistically significant. The percentage of large granule haemocytes of the 0.05 ppm exposed animals was comparable to the control.

4.4.2.1.3. Phagocytic activity

Exposure to nuvan resulted in a decrease in the phagocytic ability of the animals exposed to 0.05 ppm concentration, which was statistically significant ($p < 0.05$) (Table 11). But the phagocytic activities of 0.02 and 0.08 ppm nuvan

exposed animals were comparable with the control value. The animals in the 0.02 ppm treated group exhibited a mean phagocytic percentage of 55.66 ± 4.04%. The group exposed to 0.08 ppm nuvan gave a mean percentage phagocytosis of 55.33 ± 4.16% (Fig.13).

4.4.2.2. Humoral factors

4.4.2.2. 1. Total serum protein

The animals on exposure to different concentrations of nuvan displayed a significant decrease ($p < 0.05$) in the total serum protein concentration (Table 12). Among the treatments, the animals exposed to 0.02 ppm nuvan showed the lowest protein concentration and the animals exposed to the higher concentrations, 0.05 and 0.08 ppm nuvan registered an increase in the protein concentration which was significant ($p < 0.05$)(Fig. 14).

4.4.2.2. 2. SDS-PAGE

A discernable difference in the appearance of polypeptide fractions in the test animals was noticed on SDS-PAGE of the serum. In general, the polypeptide fractions of molecular weight above 98 kDa were more pronounced in the test groups, when compared to the control. The expression of the high molecular weight fractions was more pronounced in the 0.02 ppm exposed group. But a decrease in intensity of these protein bands, with increasing concentration of nuvan was also observed. The broadest band of molecular weight of 70 to 80 kDa, and the minor bands in the weight range 54 to 27 kDa were more pronounced in the 0.05 ppm exposed animals, compared to other test animals. The major band of 70 to 80 kDa weight was more expressed in the 0.08 ppm animals, also (Plate 33).

4.4.2.2.3. Haemagglutination

Pesticide treatments at higher concentrations resulted in the increase of haemagglutinating activity in *F. indicus*. The haemagglutination activities of the serum of treatment animals are given in Table 12. The control and 0.02 ppm

exposed animals expressed same agglutination titer, where as those exposed to 0.05 and 0.08 ppm showed significantly higher ($p < 0.05$).

4.4.2.2.4. Enzyme assays

4.4.2.2.4.1. Phenoloxidase

Phenoloxidase enzyme activity in animals exposed to nuvan, was significantly higher ($p < 0.05$) than that of the control animals (Table 12). As the nuvan concentration increased, there was a significant decrease in the phenoloxidase activity (Fig.16).

4.4.2.2.4.2. Serum acid phosphatase

Nuvan treatment resulted in a significant reduction ($p < 0.05$) of acid phosphatase concentration in the serum of test animals (Table 12). The enzyme concentration of the test animals decreased with increasing concentration of nuvan (Fig. 17).

4.4.2.2.4.3. Serum alkaline phosphatase

The serum alkaline phosphatase activity was significantly greater ($p < 0.05$) in the animals exposed to nuvan, as compared to the control (Table 12). Among the treatments, the 0.05 ppm exposed animals showed a significant drop ($p < 0.05$) in the enzyme activity. But the enzyme activity increased significantly ($p < 0.05$) in the 0.08 ppm exposed animals (Fig.18).

4.4.2.4. Histology

Antennal gland of animals exposed to 0.02 ppn nuvan exhibited exudate accumulation between the tubules (Plate 34). In some of the animals, the skeletal muscle cells of the cephalothorax showed degeneration. Hepatopancreas also showed degenerative changes such as rounding of the cells of the tubules. Exudation was noticed between the hepatic tubules (Plate 35). The gills showed oedema and accumulation of haemocytes. The haemolymph sinuses of the

Table 11. Cellular factors of *F. indicus* exposed to different concentrations of the organophosphate pesticide, nuvan

Treatments / Cellular Factors (Mean±SD)		0 ppm	0.02 ppm	0.05 ppm	0.08 ppm
Total haemocyte count ($\times 10^4$ cells/ haemolymph)		2148.33 ^{a} ± 2.19	1490.33 ^{b}± 22.09	2225.66 ^{a}± 48.05	3007.33 ^{c}± 196.87
Differential haemocyte count (%)	Hyalinocytes	21.0 ^{a} ± 1.0	21.0 ^{a} ± 2.0	28.33 ^{b} ± 1.2	22.0 ^{a} ± 2.64
	Small granule Haemocytes	68.0 ^{a} ± 1.15	71.33 ^{a} ±1.52	62.33 ^{b} ±2.33	71.0 ^{a} ± 2.64
	Large granule Haemocytes	10.0 ^{a} ± 1.0	7.33 ^{b} ± 0.57	9.33 ^{a} ± 1.15	6.33 ^{b} ± 0.57
Phagocytic activity (%)		51.33 ^{a} ± 3.21	55.66 ^{a} ±4.04	41.33 ^{b} ±2.08	55.33 ^{a} ± 4.16

Value bearing different superscripts differ significantly ($p< 0.05$)

Table 12. Humoral factors of *F. indicus* exposed to different concentrations of the organophosphate pesticide, nuvan

Treatments / Humoral Factors (Mean ± SD)	0 ppm	0.02 ppm	0.05 ppm	0.08 ppm
TSP (mg/ml)	57.33 ^{a} ± 0.28	34.0 ^{b}± 2.17	47.33 ^{c} ± 2.84	46.16 ^{c} ± 2.84
HA (reciprocal of titer value)	32.0 ± 0	32.0 ± 0	64.0 ± 0	64.0 ± 0
PO (enzyme units)	1.73 ^{a} ± 0.75	9.33 ^{b} ± 0.16	6.36 ^{c} ± 0.80	5.26 ^{d}± 0.14
SAcP (KA units)	41.96 ^{a} ± 0.90	33.06 ^{b} ± 0.41	33.7 ^{b} ± 0.85	31.36 ^{c} ± 0.15
SAlP (KA units)	2.17 ^{a} ± 0.0	2.70 ^{b} ± 0.0	2.44 ^{c} ± 0.06	3.39 ^{d} ±0.02

Value bearing different superscripts differ significantly ($p< 0.05$) TSP-Total serum protein; HA-Haemagglutination; PO-Phenoloxidase; SAcP- Serum Acid phosphatase; SAlP- Serum Alkaline phosphatase

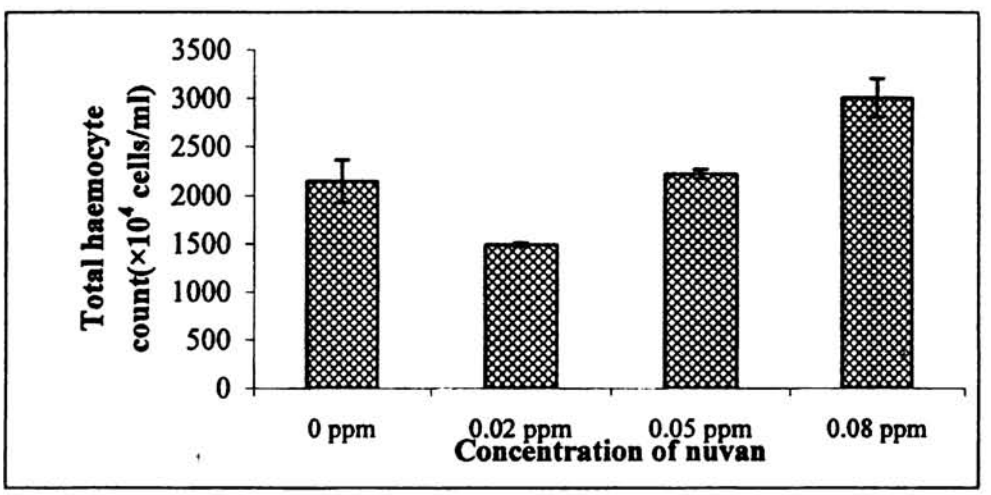

Fig. 11. Mean total haemocyte count (× 10^4 cells/ ml haemolymph) of *F. indicus* exposed to sub-lethal concentrations of organophosphate, pesticide nuvan

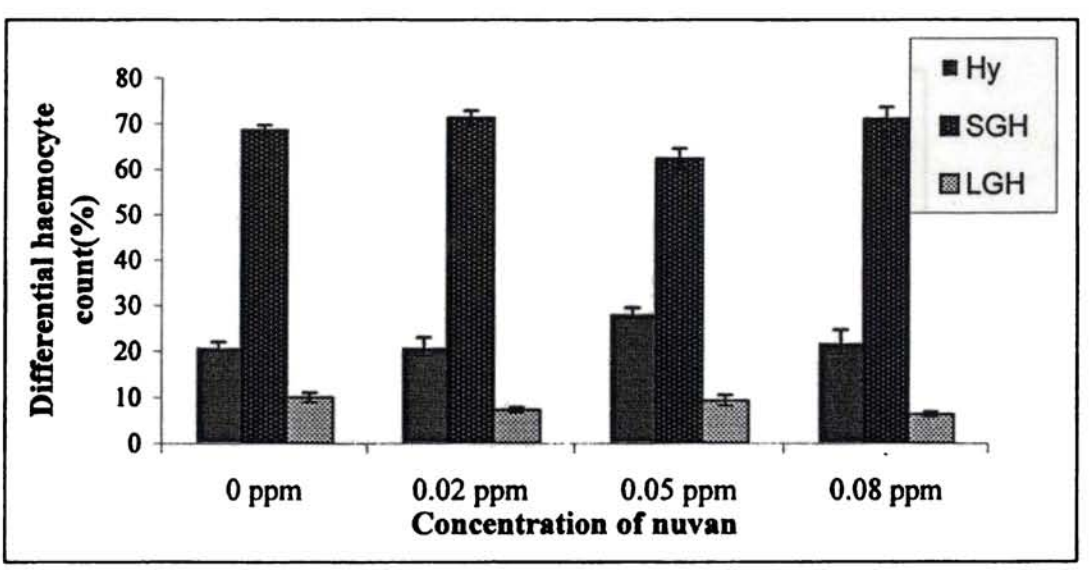

Fig. 12. Differential haemocyte count (%) of *F. indicus* exposed to different sub-lethal concentrations of organophosphate pesticide, nuvan

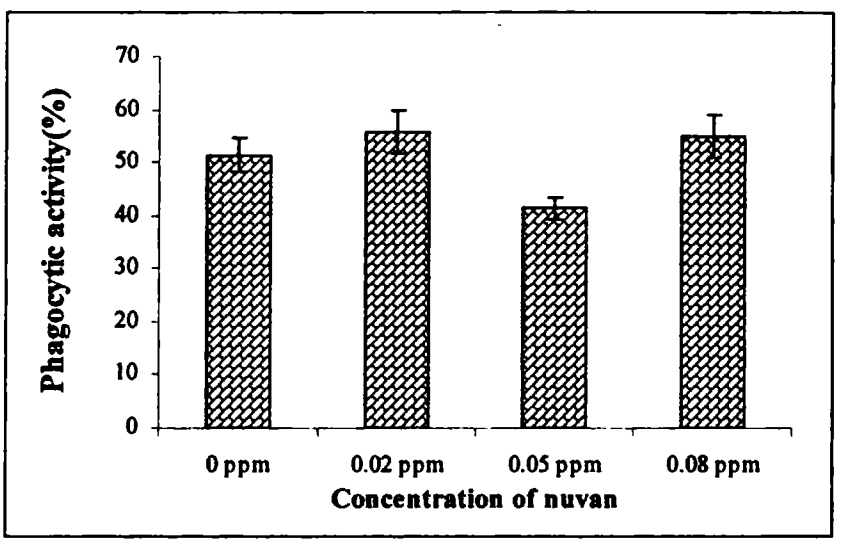

Fig. 13. Phagocytic activity (%) of the haemocytes of *F. indicus* exposed to sub-lethal concentrations of organophosphate pesticide, nuvan

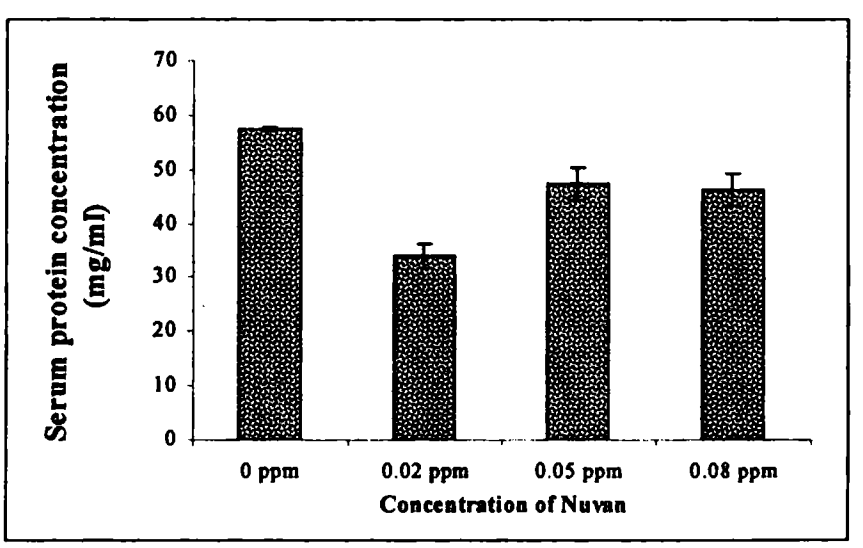

Fig.14. Mean total serum protein concentration (mg/ml) of *F. indicus* exposed to sub-lethal concentrations of the organophosphate pesticide, nuvan

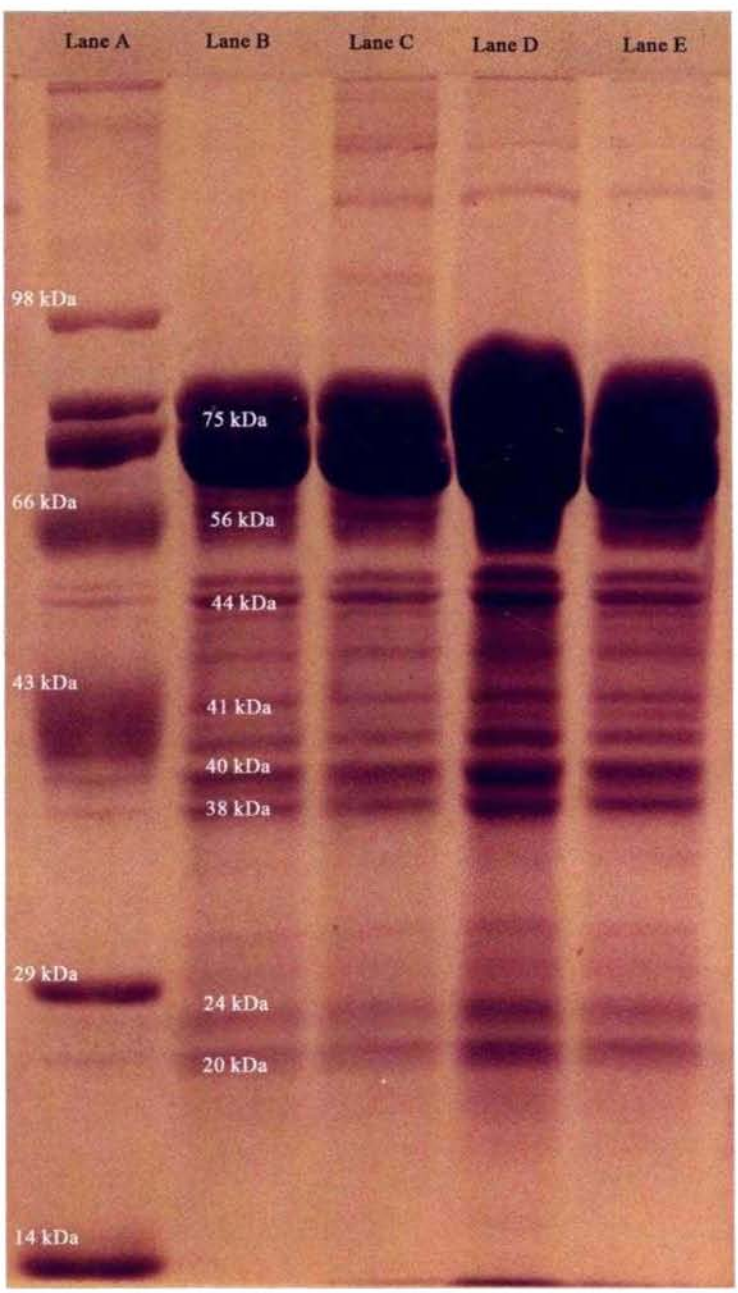

Plate 33. SDS-PAGE of the serum *F. indicus* exposed to different concentrations of organophosphate pesticide, 'nuvan'. Lane A- Molecular marker; Lane B - 0 ppm; Lane C - 0.02 ppm; Lane D - 0.05 ppm; Lane E - 0.08 ppm. Note that the fractions above 98kDA are more intense in the animals exposed to Nuvan. At 0.05 ppm (Lane D), lower molecular weight fractions are more intense than the other treatments.

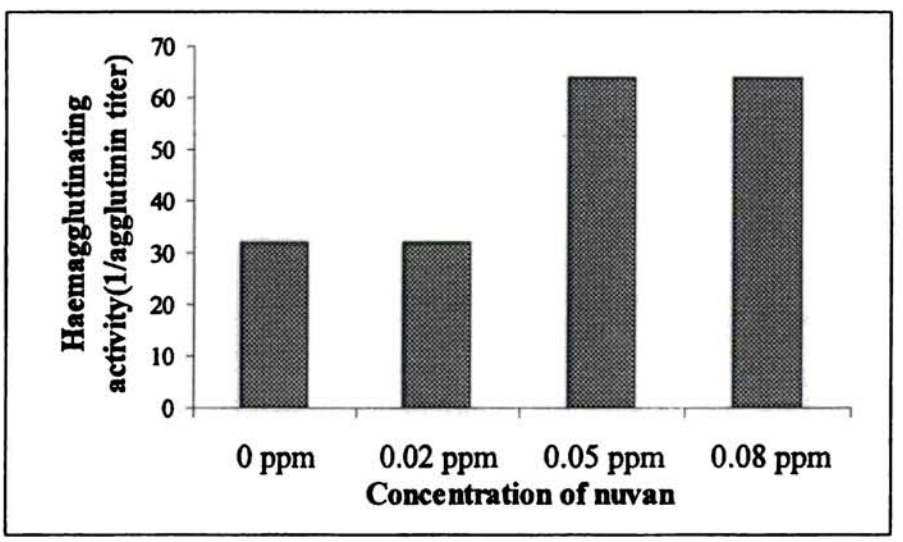

Fig. 15. Haemagglutination (1/ agglutination titer) of the serum of *F. indicus* exposed to different concentrations of the organophosphate pesticide, nuvan

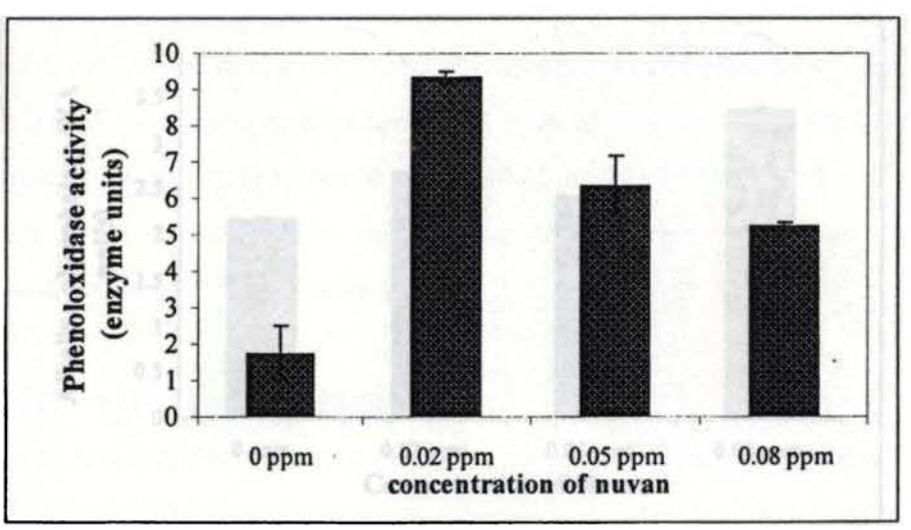

Fig.16. Phenoloxidase enzyme activity (enzyme units) of *F. indicus* exposed to different concentrations of the organophosphate pesticide, nuvan

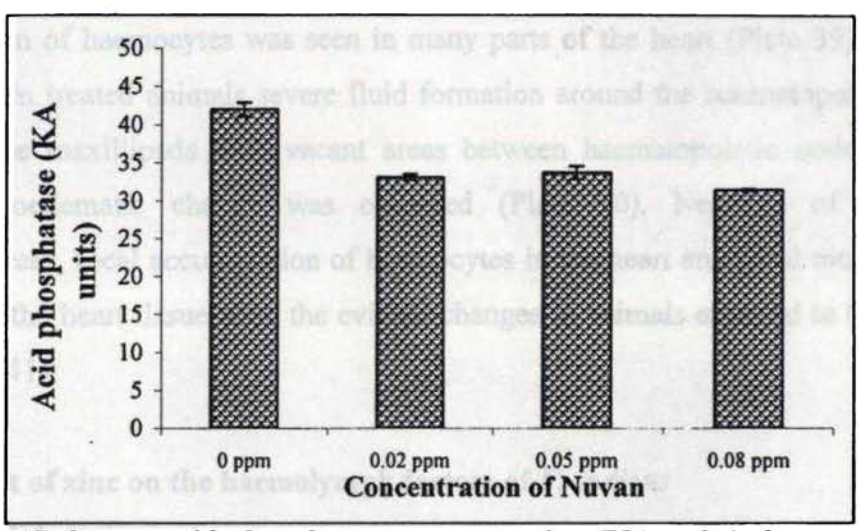

Fig 17. Serum acid phosphatase concentration (KA units)of *F. indicus* exposed to varying concentrations of organophosphate pesticide, nuvan

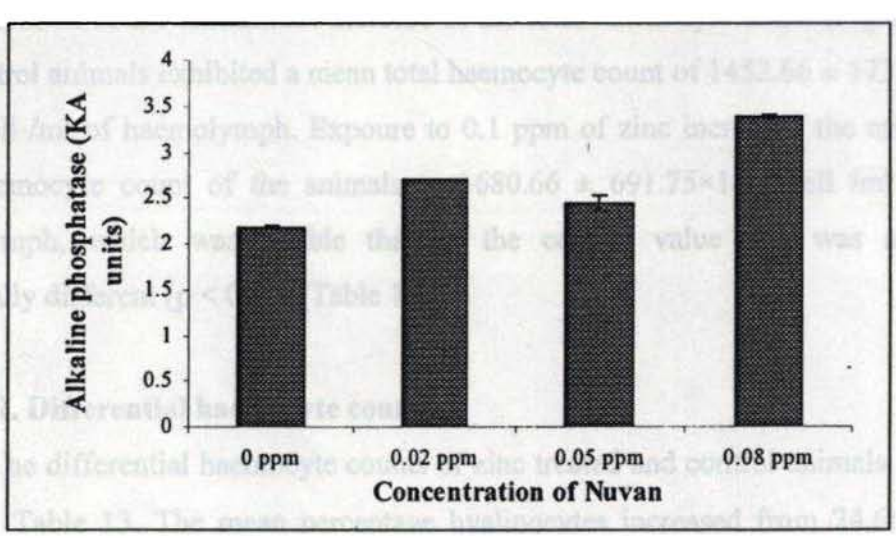

Fig. 18. Mean serum alkaline phosphatase concentrations (KA units) of *F. indicus* exposed to different concentrations of organophosphate pesticide, nuvan

secondary gill filaments were filled with haemolymph (Plate 36). Severe necrosis of the antennal gland was observed in the animals treated with 0.05 ppm nuvan. Exudation of haemolymph between the antennal tubules were also noticed (Plate 37). Hepatopancreas of the organisms was completely necrosed (Plate 38). The heart displayed acute accumulation of haemocytes and loss of striation of the myocardial bundles. Some of the heart cells also exhibited vacuolation. Focal accumulation of haemocytes was seen in many parts of the heart (Plate 39). In the 0.05 ppm treated animals severe fluid formation around the haematopoietic tissue of the maxillipeds with vacant areas between haematopoietic nodules, indicating oedematic change was observed (Plate 40). Necrosis of the hepatopancreas, focal accumulation of haemocytes in the heart and focal muscle necrosis of the heart tissue were the evident changes in animals exposed to 0.08 ppm (Plate 41).

4.4.3. Effect of zinc on the haemolymph factors of *F. indicus*

4.4.3.1. Cellular parameters

4.4.3.1.1. Total haemocyte count

Exposure of animals to sub-lethal concentration of zinc over a period of 2 weeks resulted in the remarkable increase of the total haemocyte count (Fig.19). The control animals exhibited a mean total haemocyte count of 1452.66 ± 173.20 $\times 10^4$ cell /ml of haemolymph. Expoure to 0.1 ppm of zinc increased the mean total haemocyte count of the animals to 3680.66 ± 691.75$\times 10^4$ cell /ml of haemolymph, which was double that of the control value and was also statistically different ($p < 0.05$) (Table 13).

4.4.3.1.2. Differential haemocyte count

The differential haemocyte counts of zinc treated and control animals are given in Table 13. The mean percentage hyalinocytes increased from 24.66 ± 3.51 % to 28.33 ± 2.08 %. The mean percentage of small granule haemocytes decreased from a 71 ± 4.53 % to 64 ± 2 % and that of large granule haemocytes increased from 4.66 ± 1.52 % to 8 ± 2 % (Fig 20). Although there were minor

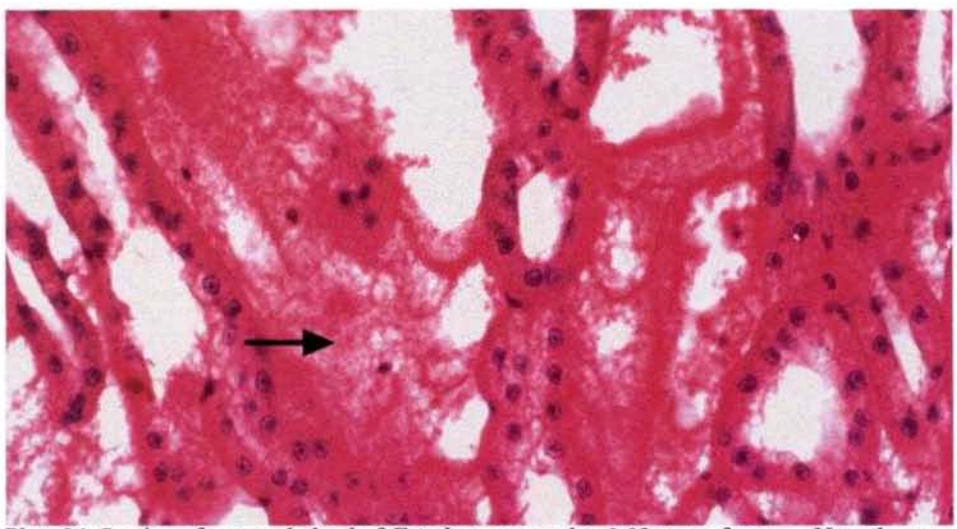

Plate 34. Section of antennal gland of *F. indicus* exposed to 0.02 ppm of nuvan. Note the exudation between the tubules (arrow). H&E stain. 400X.

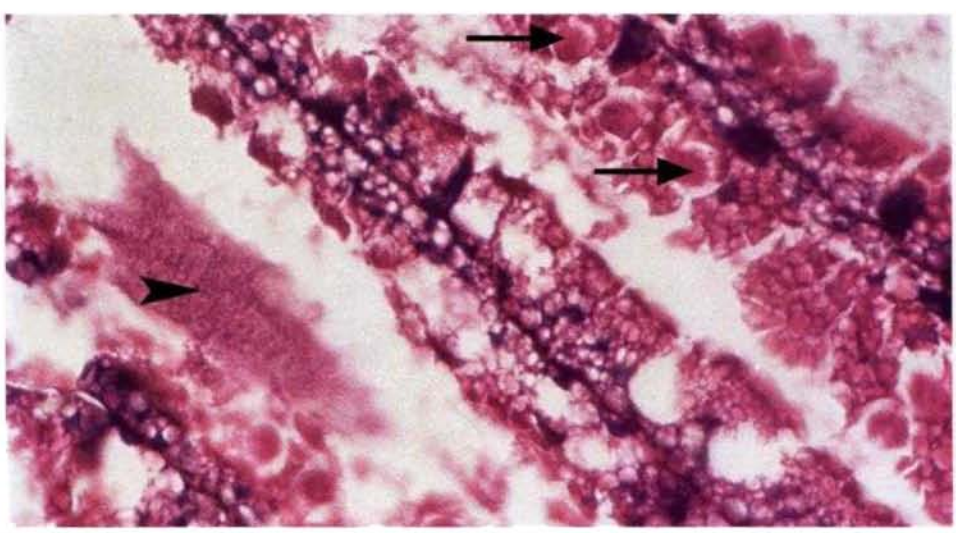

Plate 35. Section of the hepatopancreas of *F. indicus* exposed to 0.02 ppm of nuvan. Note the rounding of cells (arrow) and exudation between the hepatic tubules (arrow head). H&E stain. 400X.

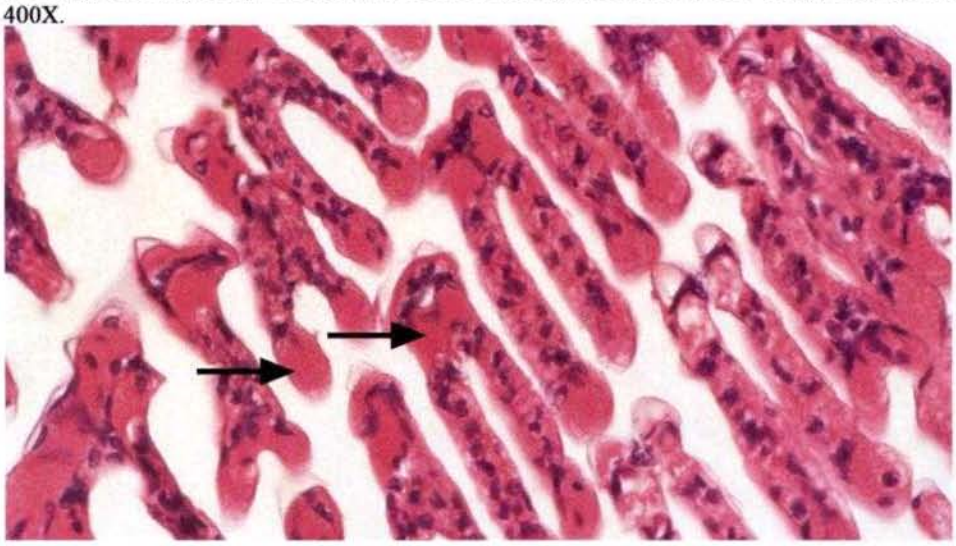

Plate 36. Section of secondary gill filaments of *F. indicus* exposed to 0.02 ppm of nuvan showing engorgement of heamal sinuses (arrow). H&E stain. 400X.

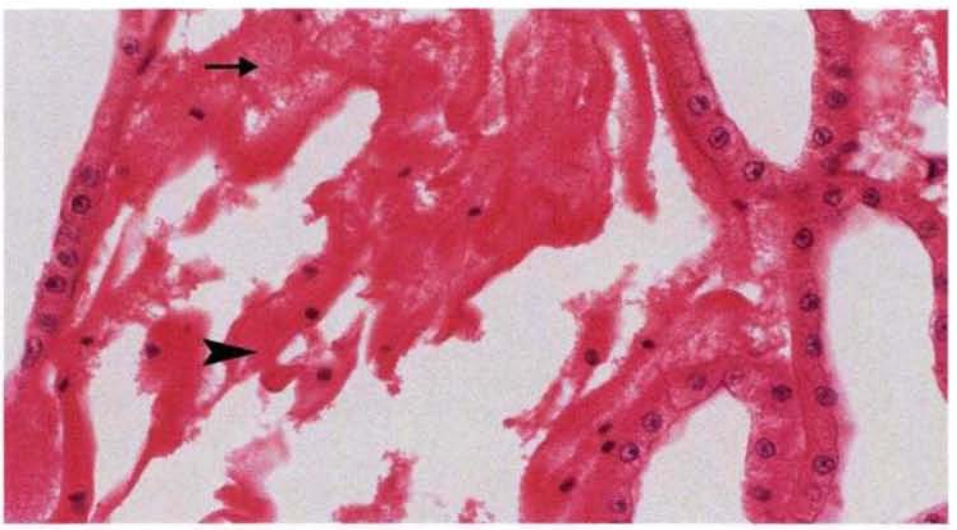

Plate 37. Section of antennal gland of *F. indicus* exposed to 0.05 ppm of nuvan. Note the exudation of heamolymph (arrow) and necrotic changes (arrow head). H&E stain. 400X.

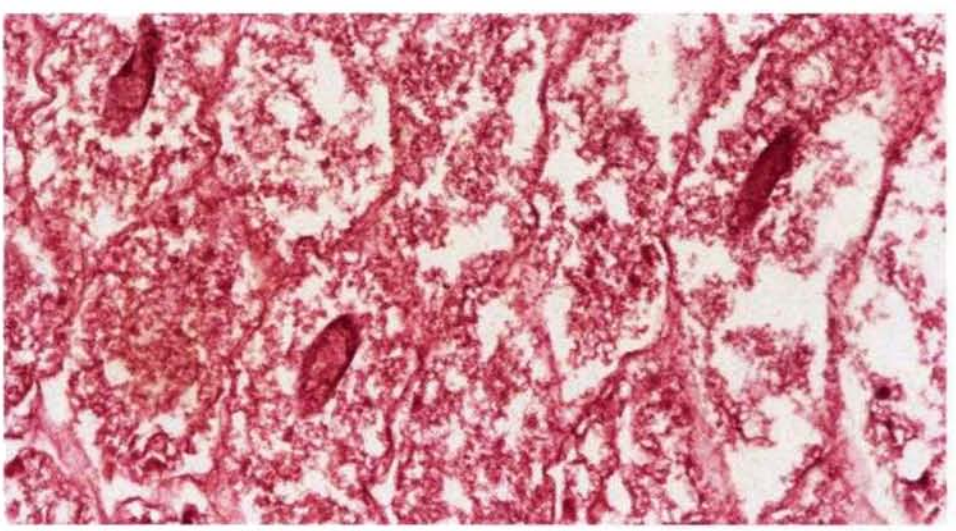

Plate 38. Section of hepatopancreas of *F. indicus* exposed to 0.05 ppm of nuvan showing complete necrosis. H&E stain. 400X.

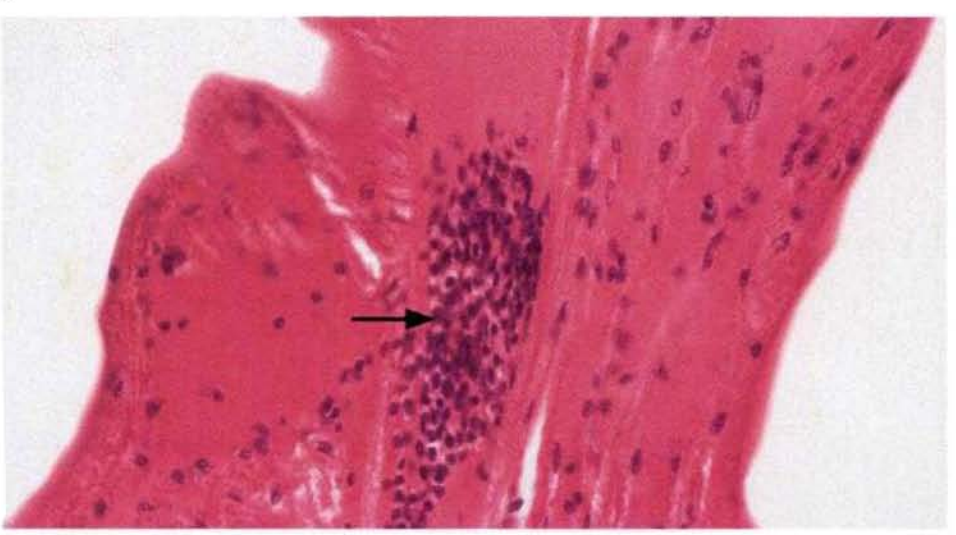

Plate 39. Section of heart of *F. indicus* exposed to 0.05 ppm of nuvan showing focal accumulation of haemocytes (arrow). H&E stain. 400X.

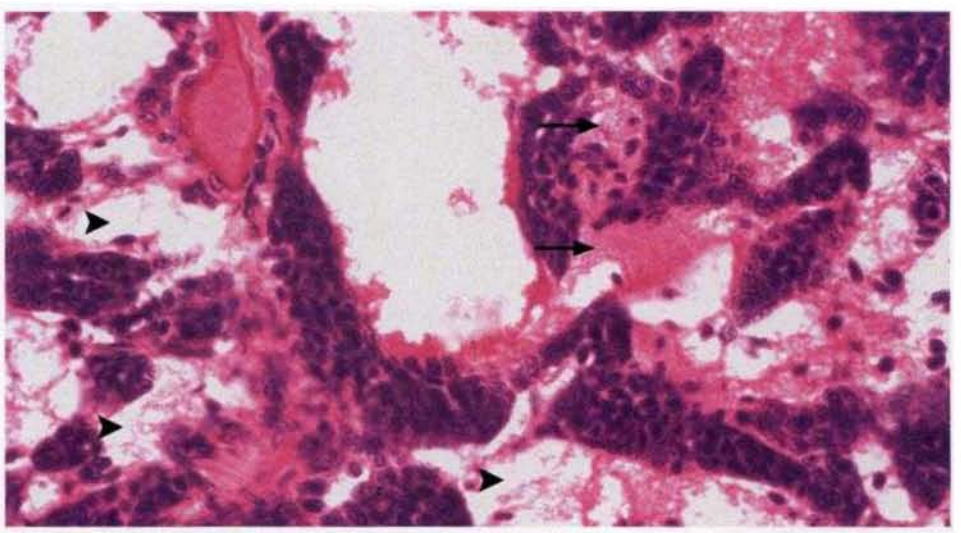

Plate 40. Section of haematopoietic tissue of *F. indicus* exposed to 0.05 ppm of nuvan. Note fluid formation (arrow) and vacant areas (arrow head) between the lobules. H&E stain. 400X.

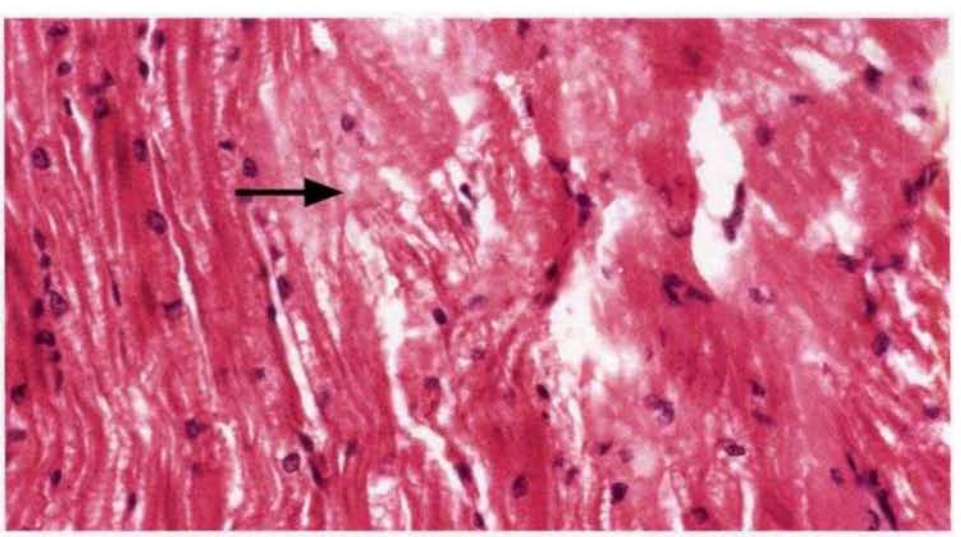

Plate 41. Section of heart of *F. indicus* exposed to 0.08 ppm of nuvan showing focal necrosis (arrow). H&E stain. 400X.

differences in the percentage of various haemocytes among exposed animals, none were significant ($p > 0.05$).

4.4.3.1.3. Phagocytic activity

The phagocytic activity of zinc exposed animals and the control is shown in the Table 13. Although the haemocytes of the test animals showed a higher phagocytic activity than the control, there was no significant difference ($p > 0.05$) (Fig.21).

4.4.3.2. Humoral factors

4.4.3.2.1. Total protein concentration

The total serum protein concentrations of the control and test are given in Table 14. There was no change in the serum protein concentration between the control and test groups (Fig. 22).

4.4.3.2. 2. SDS-PAGE

There was no difference in the appearance of the different polypeptide fraction between the control and test animals.

4.4.3.2.3 Haemagglutination

The test animals showed greater agglutinating activity than the control. The titer of the test animals was 85.33 ± 36.95, compared to 42.66 ± 18.47, of the control animals (Table 14). There was no significant difference ($p > 0.05$) between the control and test values (Fig. 23).

4.4.3.2. 4. Enzyme assays

4.4.3.2.4.1. Phenoloxidase

The phenoloxidase activity observed in the zinc exposed animals and the control is given in the Table 14. The mean enzyme activity in the test animals showed a significant decrease ($p < 0.05$) was suppressed to half of that of the control animals. The phenoloxidase activity of the test animals was 0.348 ± 0.11

Table 13. Cellular factors of *F. indicus* exposed to zinc

Cellular Factors (Mean±SD) \ Treatment		Control	Test
Total haemocyte count ($\times 10^4$ cells/ haemolymph)		1452.66 ±173.10	3680.66 ± 961.75*
Differential haemocyte count (%)	Hyalinocytes	24.66 ± 3.51	28.33 ± 2.08
	Small granule Haemocytes	71.0 ± 4.35	64.0 ± 2.0
	Large granule Haemocytes	4.66 ± 1.52	8.0 ± 2.0
Phagocytic activity (%)		63.0 ± 4.0	70.0 ± 2.64

Values with * differs significantly ($p < 0.05$).

Table 14. Humoral factors of *F. indicus* exposed to zinc

Humoral Factors (Mean±SD) \ Treatment		Control	Test
Total serum protein (mg/ ml)		68.83 ± 2.75	69.0 ± 3.46
Haemagglutination(reciprocal of titer value)		42.66 ± 18.47	85.33 ± 36.95
Enzymes	Phenoloxidase (Enzyme units)	0.709 ± 0.04	0.348 ± 0.11*
	Serum acid phosphatase (KA units)	25.48 ± 2.32	33.23 ± 5.30
	Serum alkaline phosphatase(KA units)	3.25 ±0.90	2.5 ± 0.28

Values with * differs significantly ($p < 0.05$).

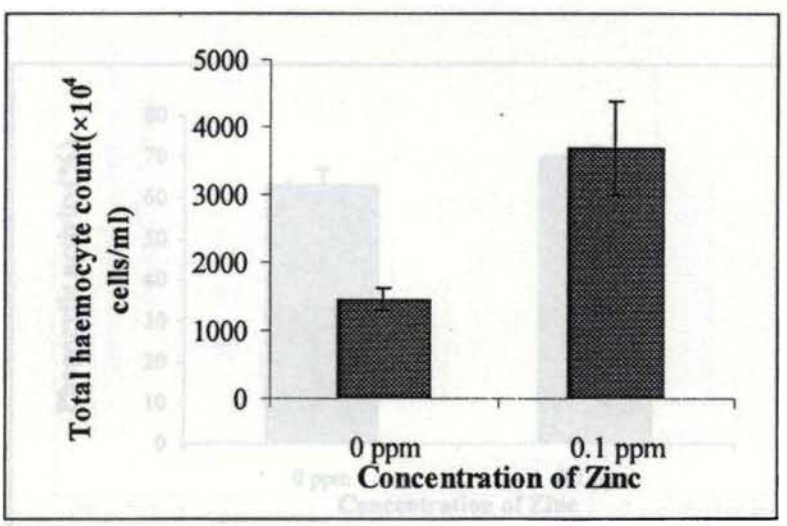

Fig 19. Total haemocyte count (× 10^4 cells/ml haemolymph) of *F. indicus* exposed to heavy metal zinc

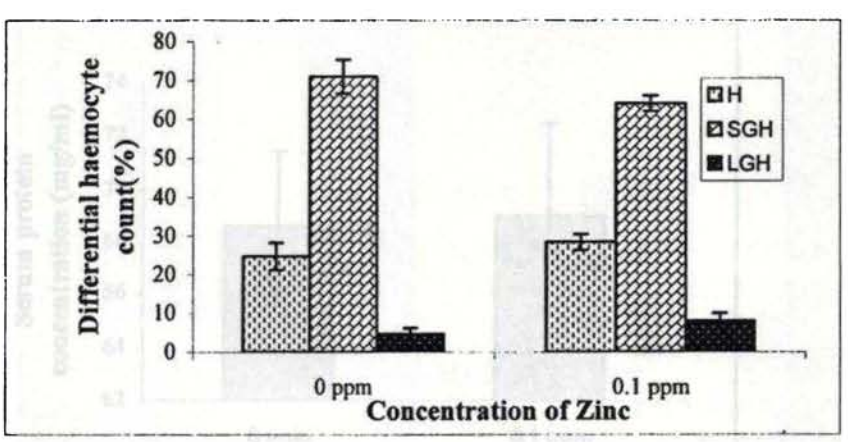

Fig. 20. Differential haemocyte count (% haemocytes) of *F. indicus* exposed to heavy metal zinc

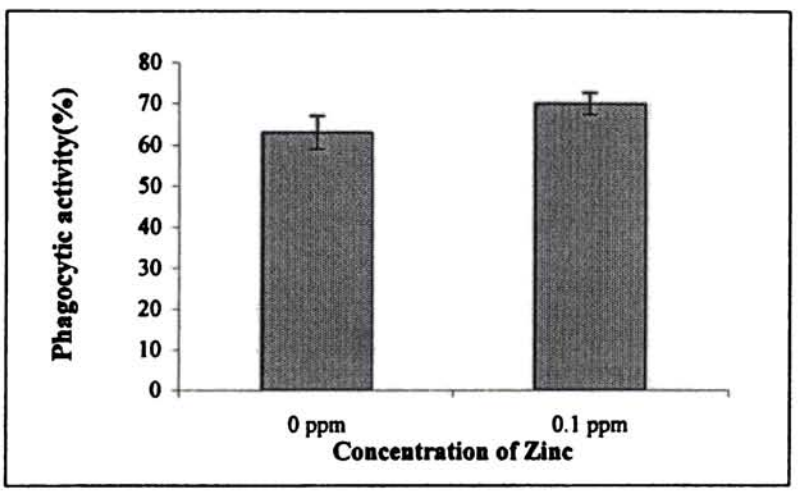

Fig. 21. Phagocytic activity of *F.indicus* exposed to heavy metal zinc

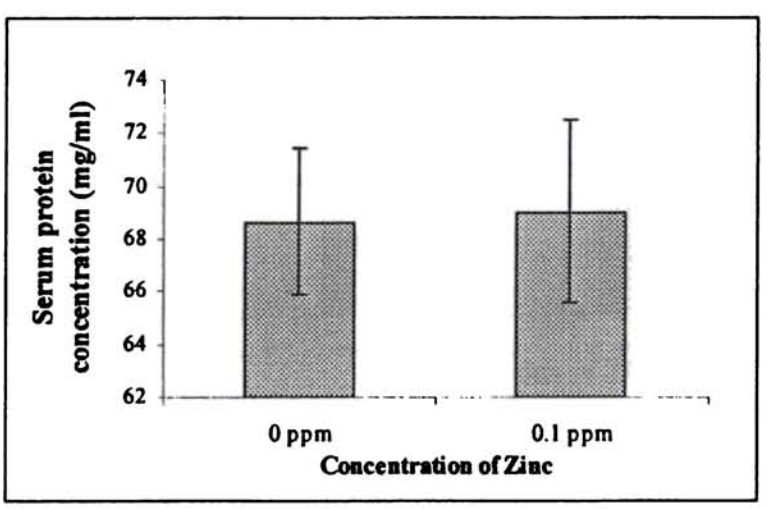

Fig. 22. Total serum protein concentration (mg/ml) of *F. indicus* exposed to heavy metal zinc

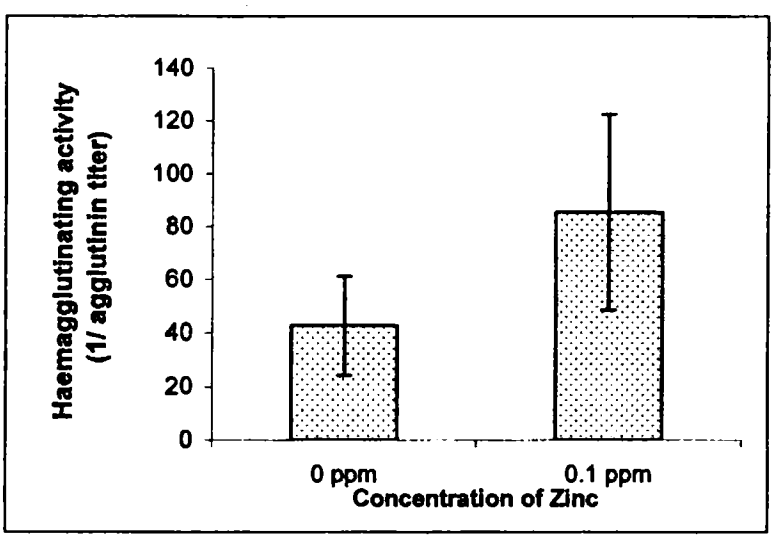

Fig. 23. Haemagglutination (reciprocal of agglutinin titer) of the serum of *F. indicus* exposed to heavy metal zinc

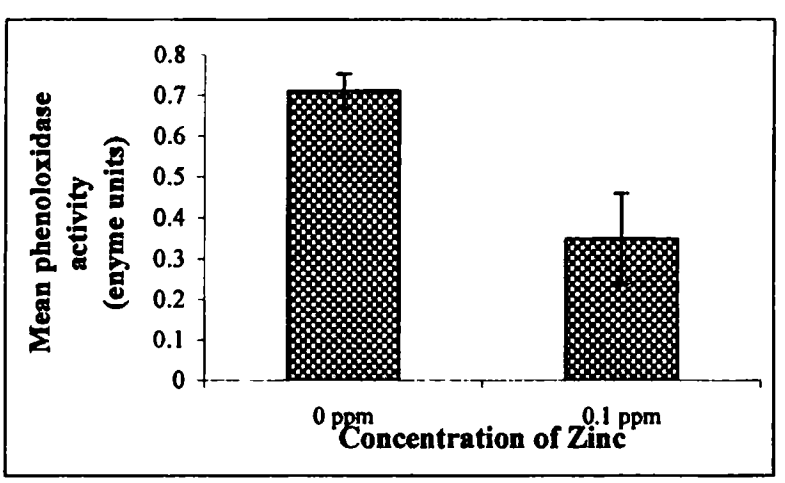

Fig. 24. Mean phenoloxidase activity (enzyme units) of *F. indicus* exposed to heavy metal zinc

enzyme units, while that of the control was 0.709 ± 0.043 enzyme units and it statistically differed ($p < 0.05$) (Fig. 24).

4.4.3.2.4.2. Acid and alkaline phosphatase

The concentration of acid phosphatase and alkaline phosphatase is given in Tables 14. The acid phosphatase activity in the serum of test animals was greater than the activity of the control animals (Fig. 25). The alkaline phosphatase enzyme activity was slightly suppressed on exposure to zinc (Fig.26). Although there were differences in enzyme activities among control and test groups, the changes were not statistically significant ($p > 0.05$).

4.4.3.4. Histology

The animals exposed to zinc resulted in variations in their tissue structure such as necrosis of the proximal part of the hepatopancreas and sloughing of the epithelial cells of the hepatic tubules (Plate 42). The gills also displayed changes *viz.* enlargement of the secondary gill filaments and of the haemal sinus of the filaments (Plate 43). The number of cells in the heart showed an increase on zinc exposure (Plate 44).

4.4.4. Effect of salinity on the haemolymph factors

4.4.4.1. Cellular factors

4.4.4.1.1. Total haemocyte Count

The animals exposed to 10 ppt salinity displayed a significant increase ($p < 0.05$) in the total haemocyte count, as compared to the animals maintained in 25 ppt (Table 15). However, in animals of 3 ppt salinity, the total haemocyte count was significantly ($p < 0.05$) lower than that of animals maintained in 25 and 10 ppt salinity (Fig. 27).

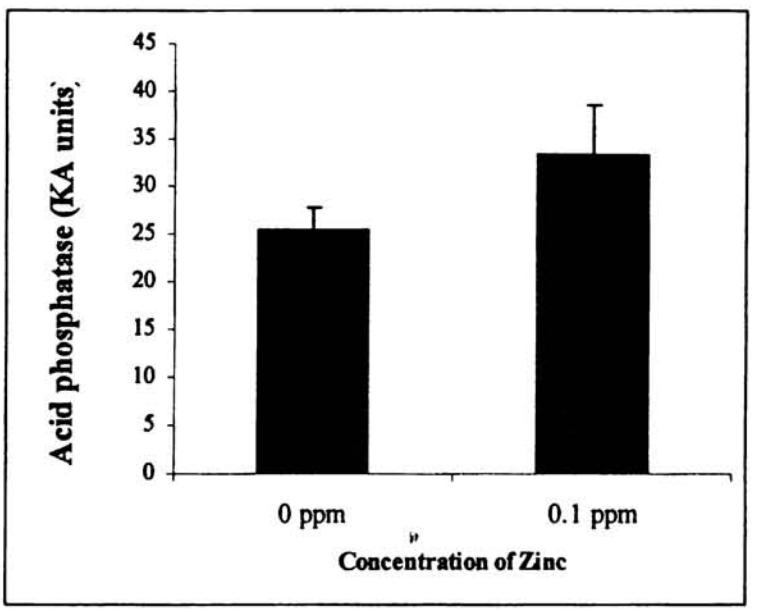

Fig. 25. Serum acid phosphatase concentrations (KA units) of *F. indicus* exposed to heavy metal zinc

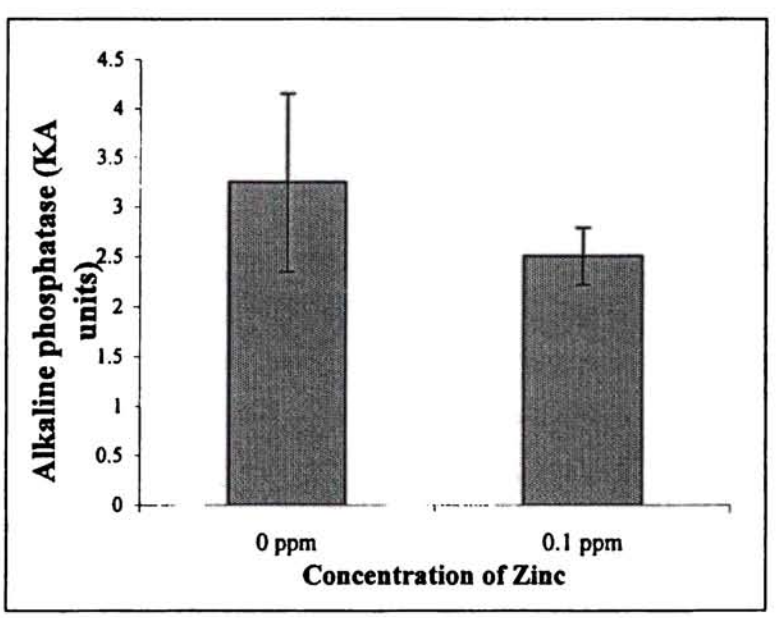

Fig. 26. Serum alkaline phosphatase activity (KA units) of *F. indicus* exposed to heavy metal zinc

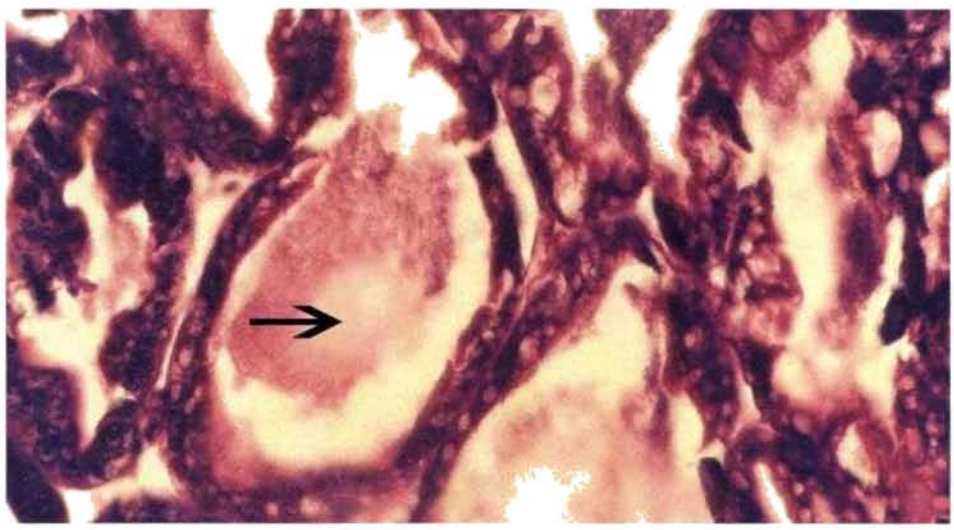

Plate 42. Section of hepatopancreas of *F. indicus* exposed to 0.1 ppm of zinc. Note the sloughing of cells in the hepatic tubules (arrow). H&E stain. 400X.

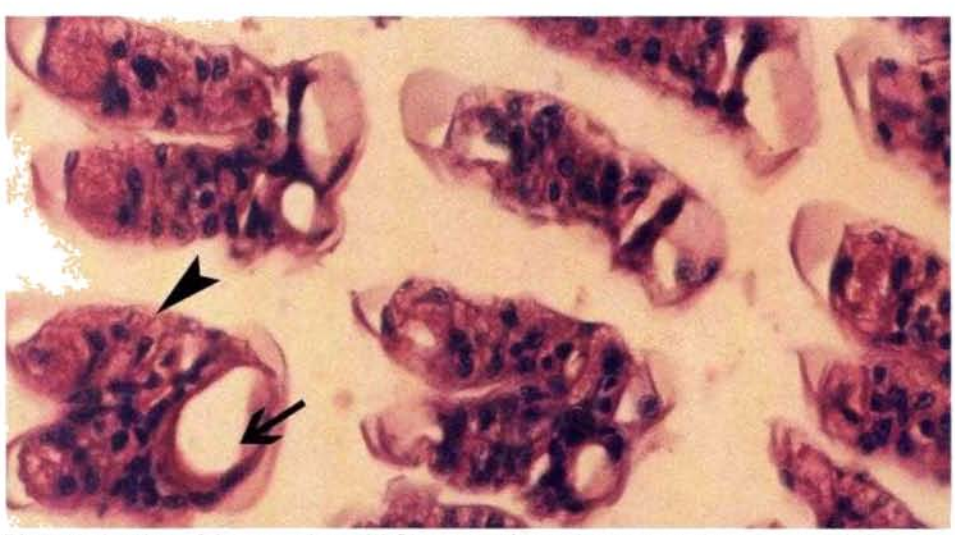

Plate 43. Section of the secondary gill filaments of *F. indicus* exposed to 0.1 ppm of zinc. Enlargement of the lacunae of gill filaments (arrow) and haemolymph sinus (arrow head) can be seen. H&E stain. 400X.

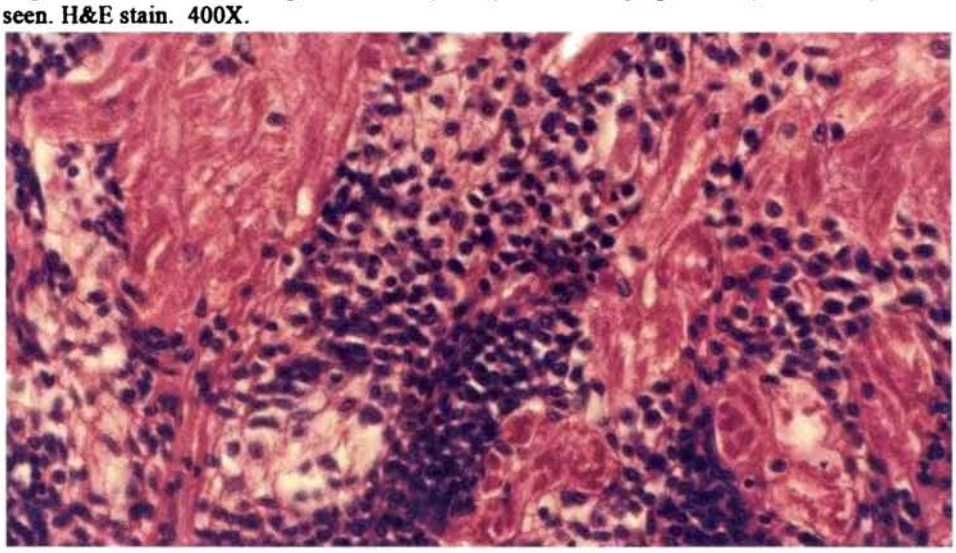

Plate 44. Section of the heart of *F. indicus* exposed to 0.1 ppm of zinc showing accumulation of haemocytes. H&E stain. 400X.

4.4.4.1.2. Differential haemocyte count

The differential haemocyte count of animals maintained in different salinities is given in Fig. 28 and Table 15.

There was a significant reduction ($p < 0.05$) in the small granule haemocytes in the animals maintained at lower salinities of 10 and 3 ppt(Fig. 28). The large granule haemocytes significantly ($p < 0.05$) increased in the animals maintained in 10 and 3 ppt salinity. The percentage of hyalinocyte in the 3 ppt salinity exposed animal was greater than that of the animals exposed to other salinities, but the difference was not statistically significant.

4.4.4.1.3. Phagocytic activity

Exposure to lower salinities caused a reduction in the phagocytic activities of the animals (Fig. 29). The 10 ppt and 3 ppt salinity exposed animals expressed a significantly lower ($p < 0.05$) phagocytic activity than those in the 25 ppt salinity (Table 15).

4.4.4.2. Humoral factors

4.4.4.2.1. Serum total protein

A reduction in serum protein concentration was observed in the animals of 10 ppt and 3 ppt salinity, which was statistically significant ($p < 0.05$) (Table 16 and Fig. 30).

4.4.4.2.2. SDS-PAGE of serum

Electrophoresis of the serum of animals exposed to three salinities showed differences in the appearance of polypeptide fractions. In the serum of animals exposed to 10 and 3 ppt salinity, fractions above 98 kDa were more intense. These were bolder in the serum of animals exposed to 10 ppt than of those exposed to 3 ppt salinity (Plate 45).

4.4.4.2.3. Haemagglutination

Exposure to low salinity produced a noticeable rise in haemagglutinating activity (Fig.31). Animals maintained in 25 ppt salinity showed an agglutinin titer of 8. In animals of both 10 and 3 ppt waters, the agglutinin titers were much higher and the difference observed was significant ($p < 0.05$) (Table 16). The shrimp in water of 10 ppt salinity displayed an agglutinin titer of 64, while the animals in 3 ppt water showed a titer of 16.

4.4.4.2.4. Enzyme assay

4.4.4.2.4.1. Phenoloxidase

Decrease in salinity resulted in the decrease of phenoloxidase activity of the animals (Fig.32), but the decrease was not statistically significant ($p < 0.05$). The enzyme activity of the animals subjected to different salinities is given in Table 16.

4.4.4.2.4.2. Serum acid phosphatase

The serum acid phosphatase level of the animals significantly ($p < 0.05$) increased with a fall in salinity (Table 16). The animals of 25 ppt salinity exhibited mean enzyme concentration of 12.19 ± 0.49 KA units. The animals exposed to lower salinities expressed higher values and the variation was statistically significant ($p < 0.05$) (Fig.33).

4.4.4.2.4.3. Serum alkaline phosphatase

The mean serum alkaline phosphatase concentration is given in Table 16. Animals maintained in 10 ppt salinity displayed a significant decrease ($p < 0.05$) in the enzyme activity. But the enzyme activity was significantly higher ($p < 0.05$) in the animals of 3 ppt, as compared to those in 25 and 10 ppt seawater (Fig.34

Table 15. Cellular factors of *F. indicus* exposed to different salinities

Cellular Factors (Mean±SD) \ Salinity		25 ppt	10 ppt	3 ppt
Total haemocyte count ($\times 10^4$ cells/ haemolymph)		1006.0 ^{a} ± 9.54	1102.0 ^{b} ± 11.27	799.33 ^{c} ± 14.5
Differential haemocyte count (%)	Hyalinocytes	27.33± 3.79	24 ± 4.58	33.67 ± 0.58
	Small-granule Haemocytes	63.67 ± 5.03 ^{a}	54.67 ^{b} ± 1.53	49.33 ^{c} ± 1.53
	Large-granule Haemocytes	12.33 ^{b} ±1.53	21.33 ^{a} ± 4.04	17 ^{a} ± 1.73
Phagocytic activity (%)		48.33 ^{a} ±2.88	35.66 ^{b} ± 2.08	34.66 ^{b} ± 3.05

Values with different superscript differs significantly ($p < 0.05$).

Table 16. Homoral factors of *F. indicus* exposed to different salinities

Humoral factors (Mean±SD) \ Salinity	25 ppt	10 ppt	3 ppt
Total serum protein (mg/ml)	57.33 ^{a} ± 0.57	33.33 ^{b} ± 0.57	32.66 ^{b} ± 0.57
Haemagglutination (reciprocal of titer value)	8.0± 0	64.0±0	16.0±0
Phenoloxidase activity (enzyme units)	1.84 ± 0.63	1.23 ± 0.1	0.8 ± 0.1
Serum acid phosphatase (KA units)	12.19 ^{a} ± 0.49	14.20 ^{b} ± 0.17	15.06 ^{c} ± 0.36
Serum alkaline phosphatase(KA units)	2.10 ^{a} ± 0.04	1.29 ^{b} ± 0.10	2.66 ^{c} ± 0.04

Values with different superscript differs significantly ($p < 0.05$).

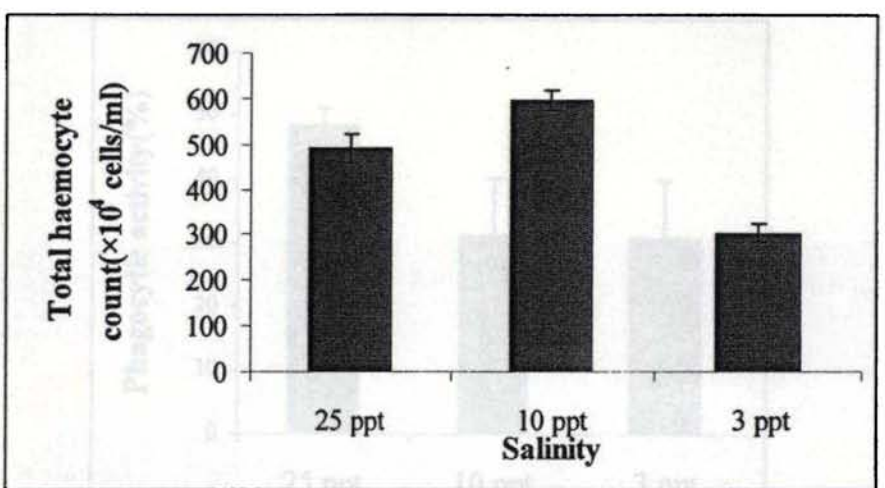

Fig. 27. Total haemocyte count (× 10^4 cell/ml haemolymph) of *F. indicus* exposed to different salinities

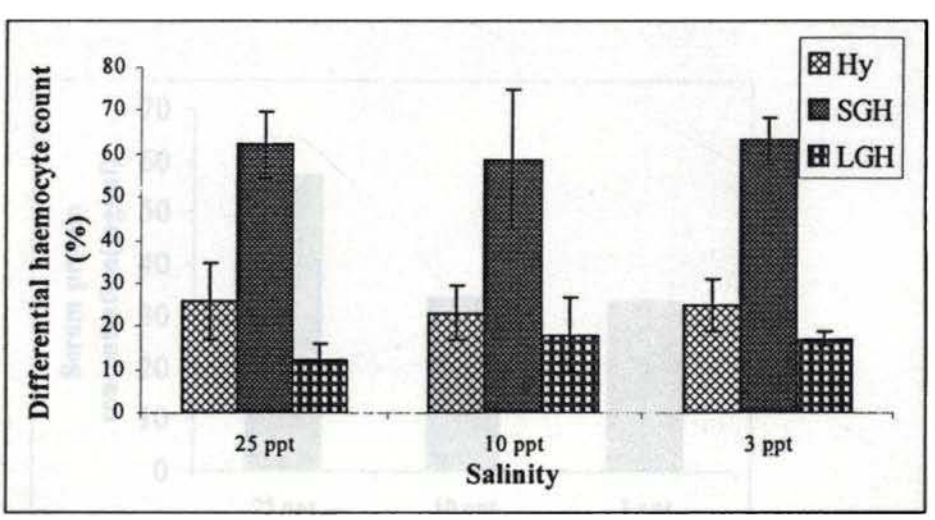

Fig. 28. Differential haemocyte count (%) of *F. indicus* exposed to different salinities

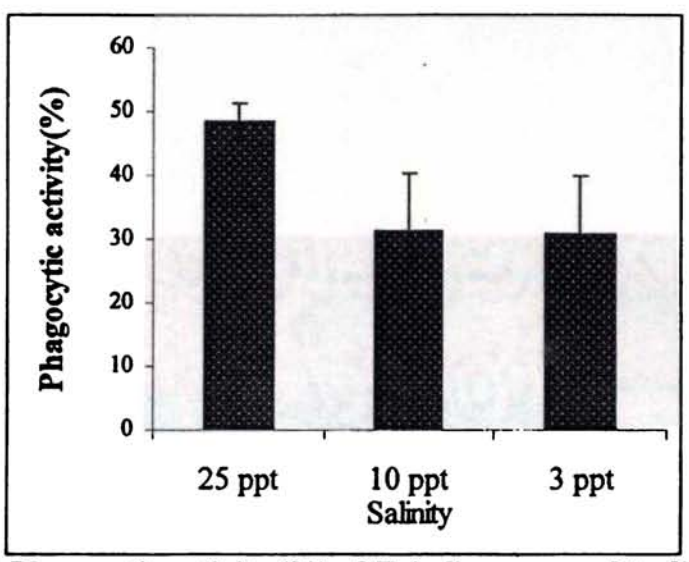

Fig. 29. Phagocytic activity (%) of *F. indicus* exposed to different salinities

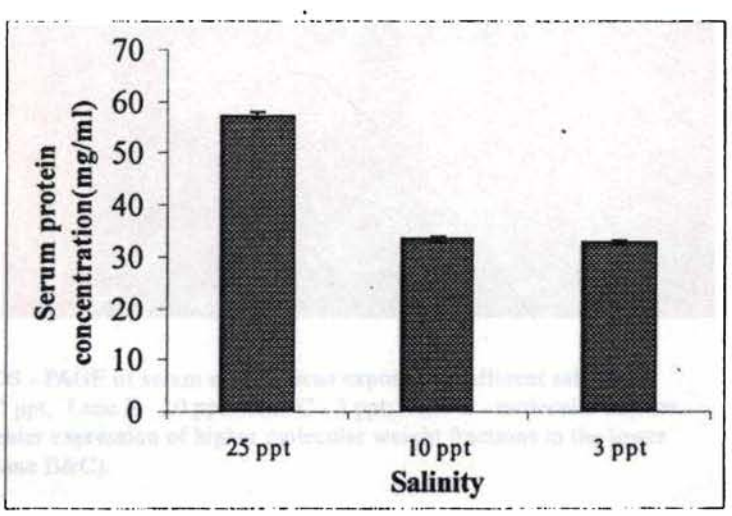

Fig. 30. Total serum protein concentration (mg/ml haemolymph) of *F. indicus* exposed to different salinities

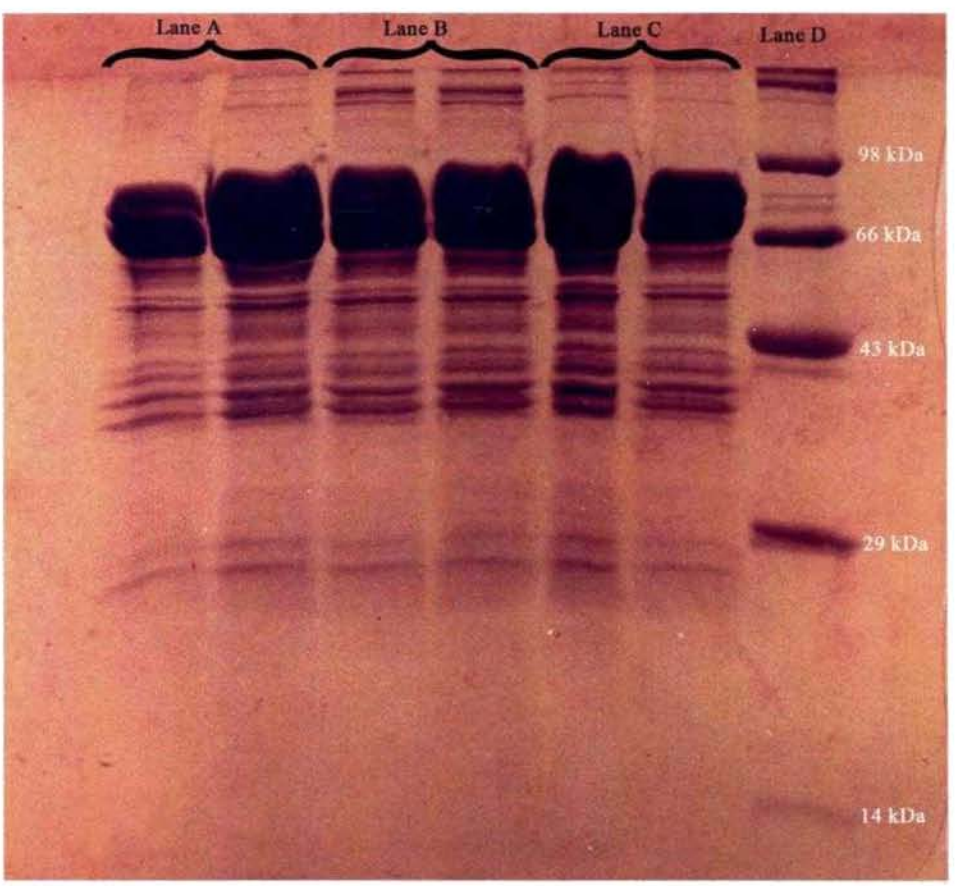

Plate 45. SDS - PAGE of serum of *F. indicus* exposed to different salinities. Lane A - 25 ppt; Lane B - 10 ppt; Lane C - 3 ppt; Lane D - molecular marker. Note the greater expression of higher molecular weight fractions in the lower salinities (Lane B&C).

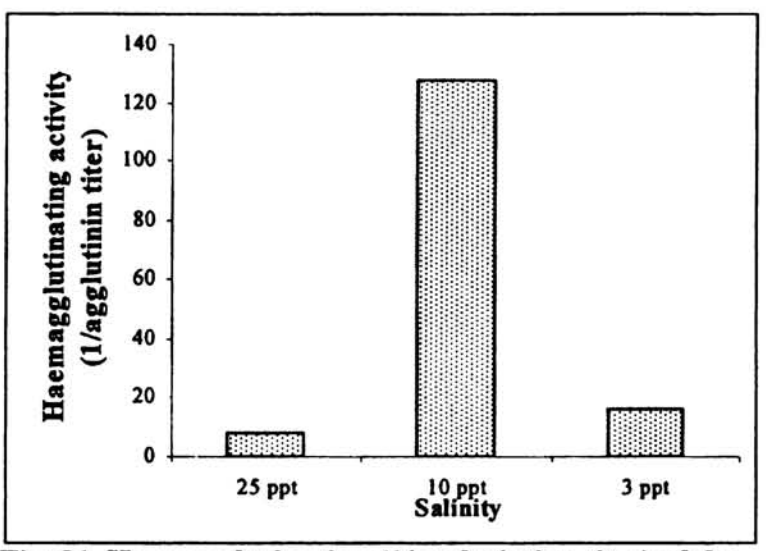

Fig. 31. Haemagglutination (1/agglutiation titer) of the serum of *F. indicus* exposed to different salinities

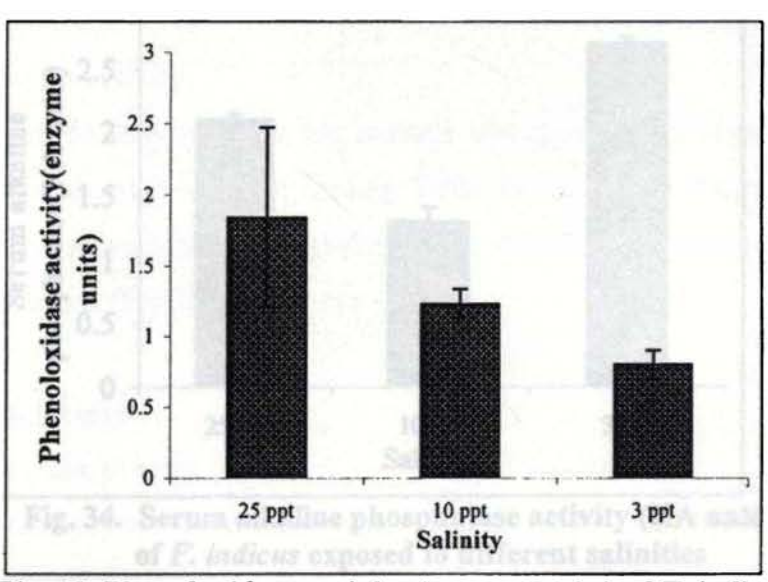

Fig. 32. Phenoloxidase activity (enzyme units) of *F. indicus* exposed to different salinities

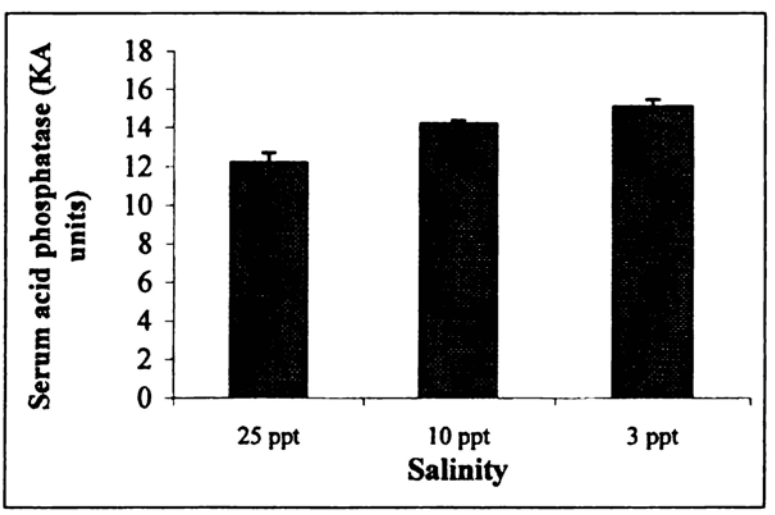

Fig. 33. Serum acid phosphatase (KA units) of *F. indicus* exposed to different salinities

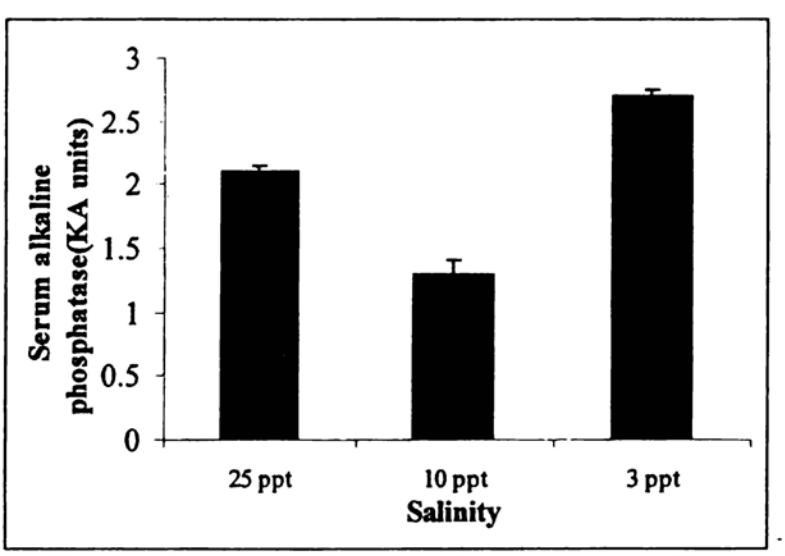

Fig. 34. Serum alkaline phosphatase activity (KA units) of *F. indicus* exposed to different salinities

4.4.5. Effect of commercial immunostimulant, 'Allways' on *F. indicus*

4.4.5.1. Cellular factors

4.4.5.1.1. Total haemocyte count

The control and test animals showed an increasing trend in the total haemocyte count, but the values of the test animals was lower than the control animals (Fig.35). At 4 weeks, the total haemocyte count of the test animals significantly increased ($p < 0.05$) than the control (Table 17).

4.4.5.1.2. Differential haemocyte Count

The differential haemocyte count of the control and test shrimps is given in the Table 17. At 4 weeks of feeding immunostimulamt incorporated feed, the percentage of hyalinocytes was significantly greater than the control (Fig.36). The percentage of small granule haemocytes was significantly greater ($p < 0.05$) than the control, initially and at 4 weeks (Fig.37). The percentage of large granule haemocytes increased significantly in the test animals at 1 week. The percentage of this haemocyte expressed a reduction at 3 weeks, but increased at 4 weeks (Fig.38).

4.4.5.1.3. Phagocytic activity

The phagocytic activity of the test animals was significantly lower ($p < 0.05$) than the control, at 1 week of feeding Table 17. At 3 and 4 weeks of experiment, the test animals showed significantly ($p < 0.05$) greater phagocytic activity than the control (Fig.39).

4.4.5.2. Humoral factors

4.4.5.2.1. Total serum protein

Total serum protein concentration of animals given immunostimulant added feed and normal feed are given in the Table 18. The serum protein concentration of the control and test animals showed the same trend, during the experimental period ($p < 0.05$). Total serum protein was less in test animals, as compared to control animals (Fig 40), and was significantly so at 2 weeks and 4 weeks.

Table 17.Cellular factors of *F. indicus* fed immunostimulant incorporated feed during different time intervals

Time intervals / Cellular factors			0 week	1 week	2 weeks	3 weeks	4 weeks
THC($\times 10^4$ cells/ml)	Control	Mean ±SD	1624.66 ±201.2	1838.66 ±161.57	4085.33 ±224.64	5300.0 ±35.67	2412.0 ±109.88
	Test		1515.0 ±53.69	1771.66 ±126.53	3372.0 ±84.66	4153.66 ±315.59	3554.66* ±168.22
% Hy	Control	Mean ±SD	17.0±2.64	22.33±2.08	15.33±3.78	20.66±5.5	20.0±1.0
	Test		20.0±2.0	21.0±4.35	16.33±3.05	23.0±6.08	23.66* ±1.52
%SGH	Control	Mean ±SD	66.0±0	71.0±1.0	77.0±4.0	60.66±8.33	56.66±2.08
	Test		67.66* ±0.57	69.0±5.19	74.66±2.08	68.33±8.96	62.66* ±2.08
%LGH	Control	Mean ±SD	5.0±0	4.66±0.57	7.33±2.51	18.33±1.52	23.33±2.08
	Test		4.66±0.57	10.0* ±1.0	8.66±1.15	9.0* ±2.64	14.0* ±2.64
%Phag	Control	Mean ±SD	53.0±3.6	72.66±6.65	60.66±8.62	42.66±3.21	44.0±1.73
	Test		52.66±5.68	48.66* ±9.07	57.33±3.31	53.33* ±3.21	50.33* ±1.52

Values with asterix(*) differ from their contemporary control values by a significance level of 95%

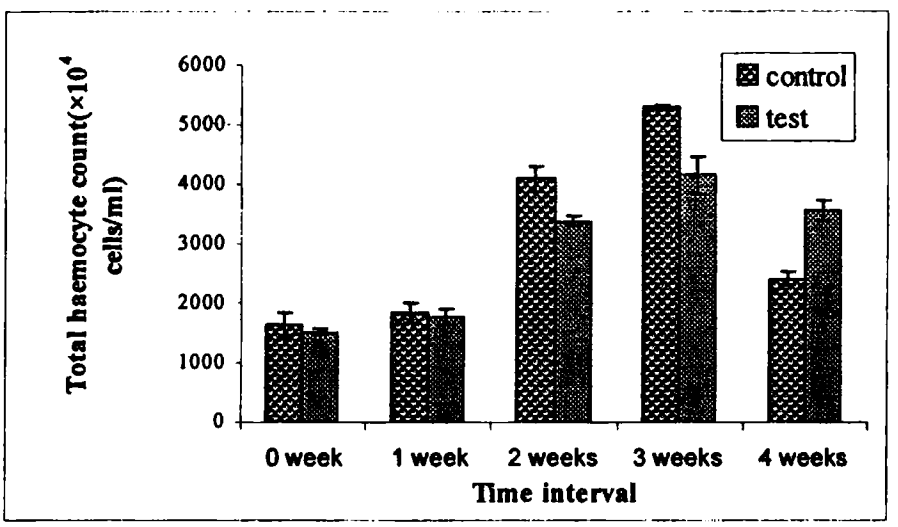

Fig. 35. Total haemocyte count (×10^4 cells/ml haemolymph) of *F. indicus* fed immunostimulant incorporated feed during different time intervals

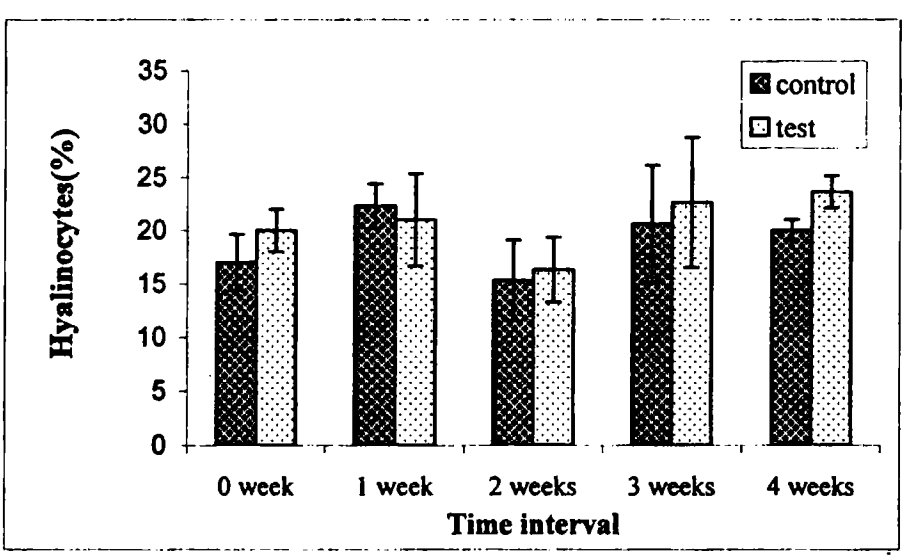

Fig. 36. Hyalinocytes (%) of *F. indicus* fed immunostimulant incorporated feed during different time intervals

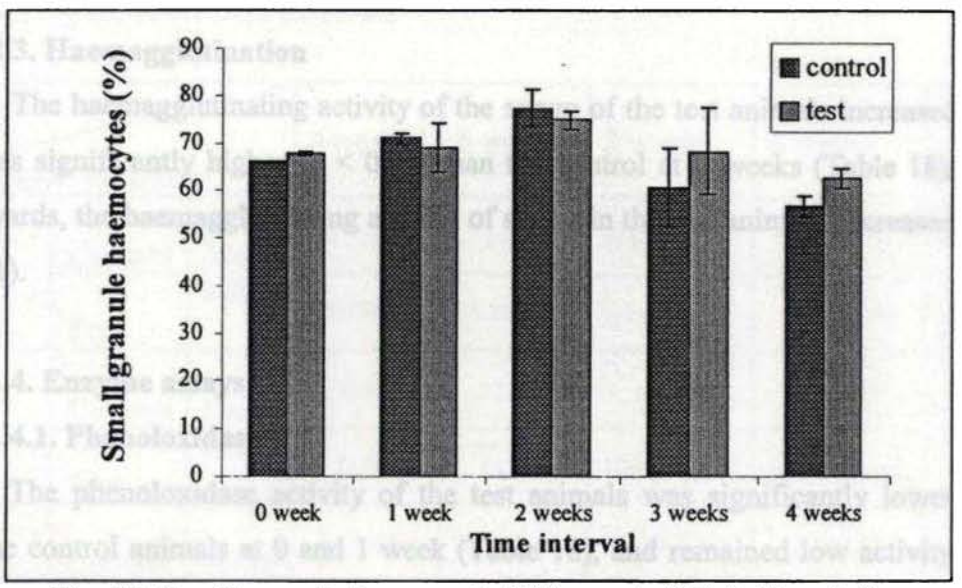

Fig. 37. Small granule haemocyte (%) count of *F. indicus* fed immunostimulant incorporated feed during different time intervals

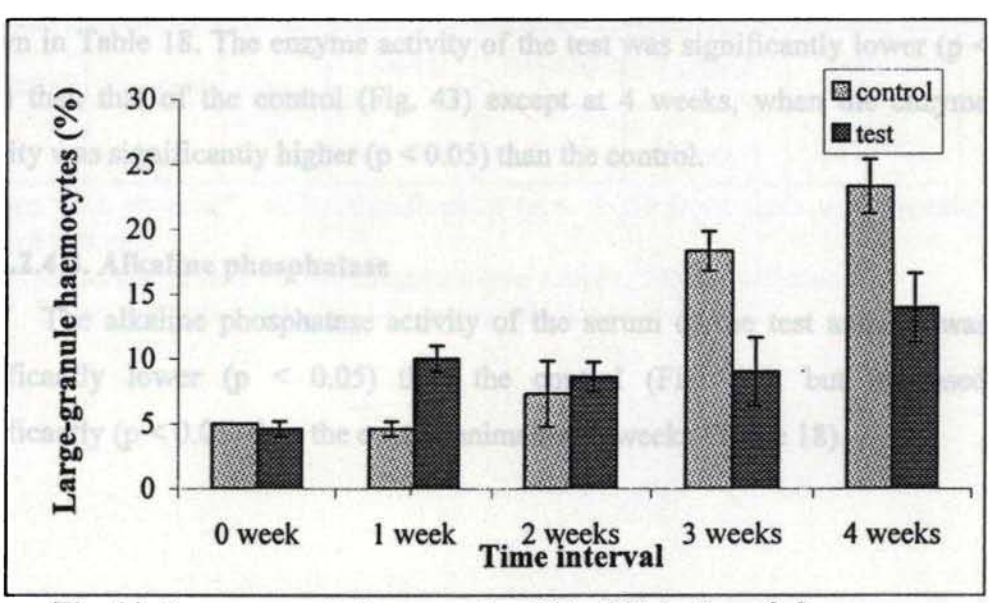

Fig. 38. Large granule haemocytes (%) of *F. indicus* fed immunostimulant incorporated fed during different time intervals

4.4.5.2.2. SDS-PAGE

Electrophoresis did not show any noticeable difference among the different polypeptide fractions between the control and test animals.

4.4.5.2.3. Haemagglutination

The haemagglutinating activity of the serum of the test animals increased and was significantly higher ($p < 0.05$) than the control at 3 weeks (Table 18). Afterwards, the haemagglutinating activity of serum in the test animals decreased (Fig.41).

4.4.5.2.4. Enzyme assays

4.4.5.2.4.1. Phenoloxidase

The phenoloxidase activity of the test animals was significantly lower than the control animals at 0 and 1 week (Table 18), and remained low activity for the rest of the experiment (Fig. 42).

4.4.5.2.4.2. Serum acid phosphatase

Mean acid phosphatase enzyme levels of control and test animals are shown in Table 18. The enzyme activity of the test was significantly lower ($p < 0.05$) than that of the control (Fig. 43) except at 4 weeks, when the enzyme activity was significantly higher ($p < 0.05$) than the control.

4.4.5.2.4.3. Alkaline phosphatase

The alkaline phosphatase activity of the serum of the test animals was significantly lower ($p < 0.05$) than the control (Fig. 44), but increased significantly ($p < 0.05$) than the control animals at 3 weeks (Table 18).

Table 18. Humoral factors of *F. indicus* fed immunostimulant incorporated feed during different time intervals

Time intervals / Humorl factors			0 week	1 week	2 weeks	3 weeks	4 weeks
TSP (mg/ml)	Control	Mean ±SD	54.77±1.76	52.0±1.5	51.16±0.28	54.16±0.76	58.33±0.57
	Test		53.07±1.95	49.66±0.76	49.16* ±0.28	54.66±0.28	53.83* ±0.57
HA (reciprocal of titer)	Control	Mean ±SD	32.0±0	32.0±0	64.0±0	32.0±10	21.33±9.24
	Test		32.0±0	64.0±0	106.66±36.95	106.66* ±36.95	26.67±9.24
PO(enzyme units)	Control	Mean ±SD	0.85±0.05	1.04±0.07	0.57±0.11	0.6±0.27	0.6±0.12
	Test		0.73* ±0.04	0.54* ±0.18	0.51±0.19	0.86±0.03	0.56±0.14
SAcP(KA Units)	Control	Mean ±SD	33.15±0.21	52.5±1.08	54.39±4.62	32.4±0.46	38.34±2.26
	Test		34.95* ±0.18	47.61* ±0.21	43.23* ±1.5	29.29* ±0.75	45.09* ±2.27
SAlP(KA Units)	Control	Mean ±SD	1.67±0.11	1.69±0.22	7.37±0.28	4.11±0.13	4.24±0.44
	Test		1.14* ±0.12	1.33±0.1	5.22* ±0.14	5.52* ±0.28	4.74±1.03

Values with asterix(*) differ significantly ($p < 0.05$) from their contemporary control values .

TSP-Total serum protein; HA-Haemagglutinating activity; PO-Phenoloxidase; SAcP- Serum Acid phosphatase; SAlP- Serum Alkaline phosphatase

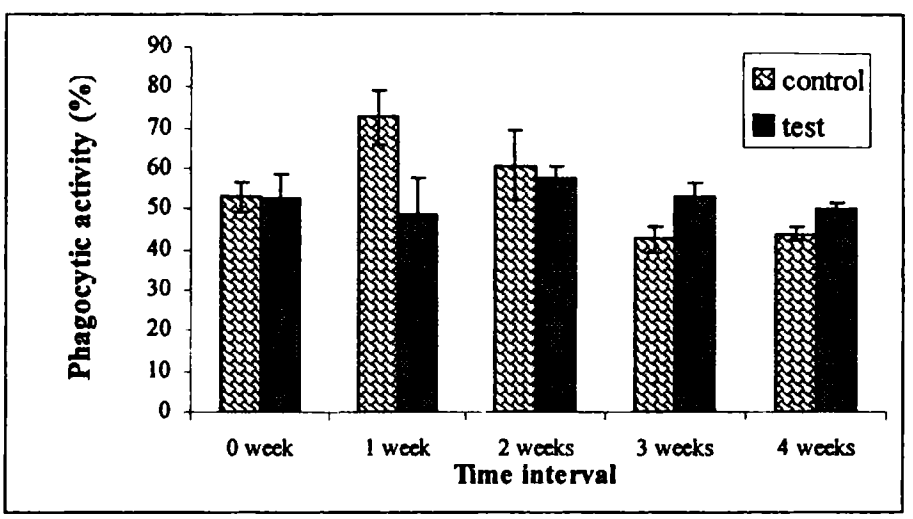

Fig. 39. Phagocytic activity (%) of *F. indicus* fed immunostimulant incorporated feed during different time intervals

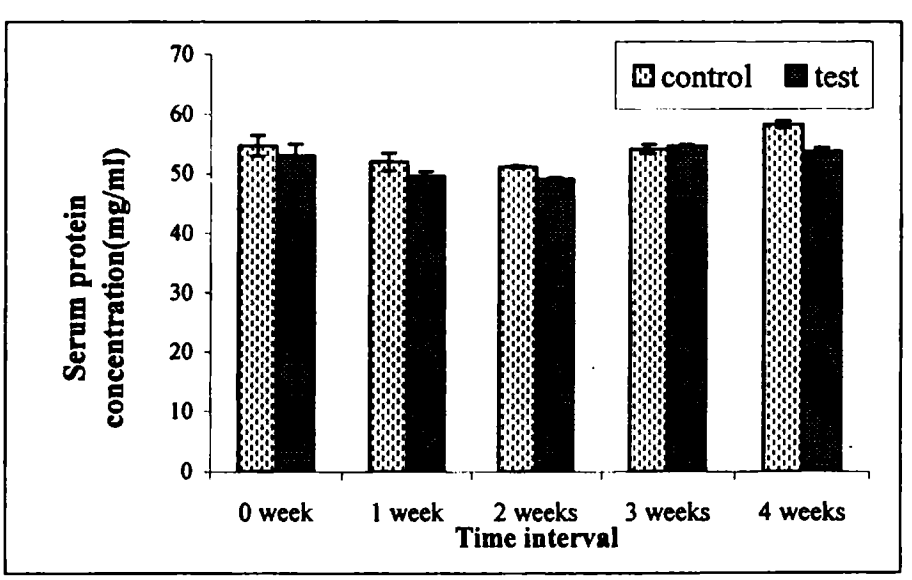

Fig. 40. Total serum protein concentration (mg/ml haemolymph) of *F. indicus* fed immunostimulant incorporated feed during different time intervals

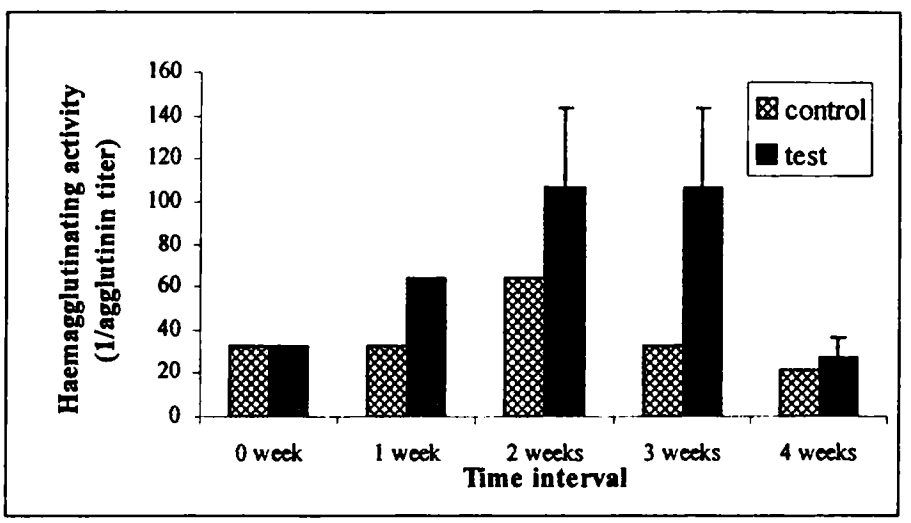

Fig. 41. Haemagglutinating activity (1/agglutinin titer) of serum of *F.indicus* fed immunostimulant incorporated feed during different time intervals

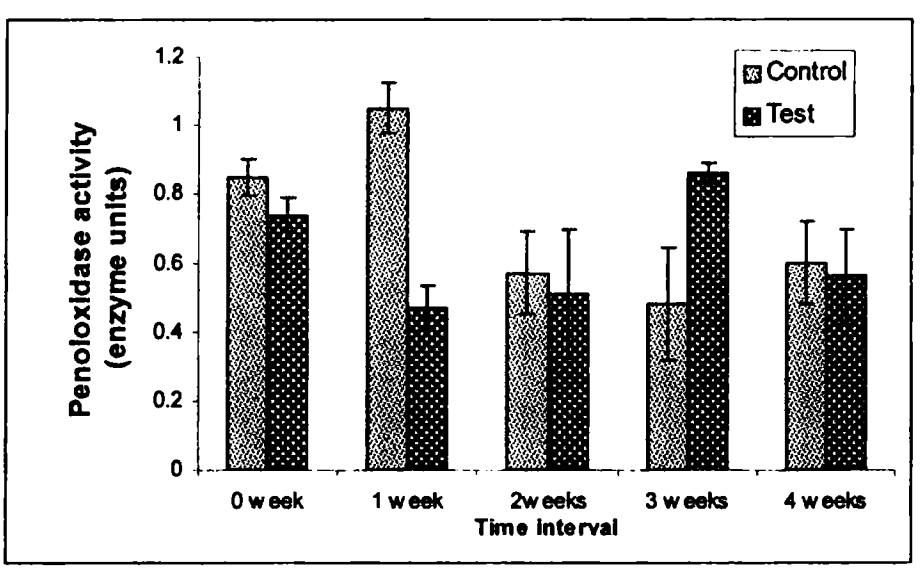

Fig. 42. Phenoloxidase activity (enzyme units) of *F. indicus* fed immunostimulant incorporated feed during different time intervals

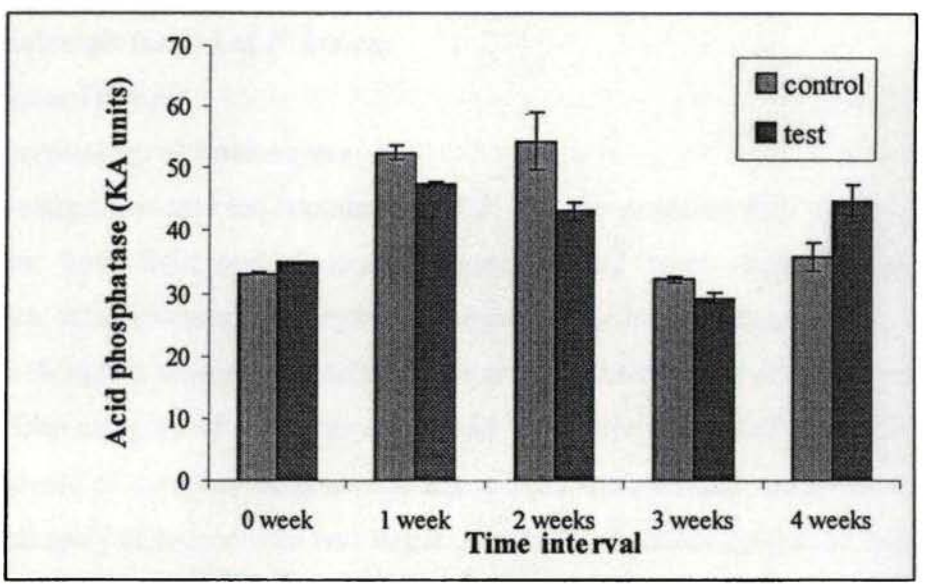

Fig. 43. Serum acid phosphatase activity (KA units) of *F. indicus* fed immunostimulant incorporated feed during different intervals

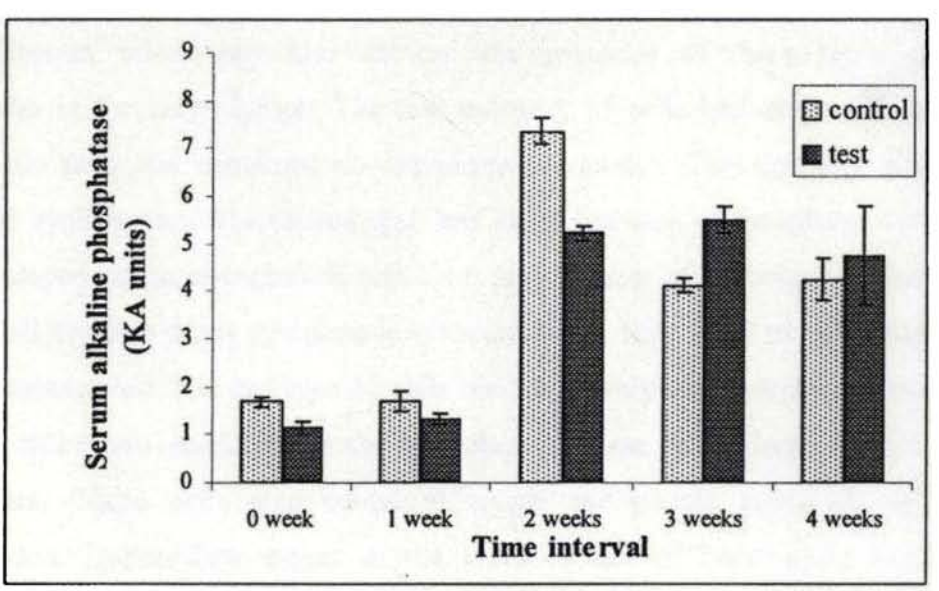

Fig. 44. Serum alkaline phosphatase activity (KA units) of *F. indicus* fed immunostimulant incorporated feed during different intervals

Discussion

5. Discussion

5.1. Haemolymph factors of *F. indicus*

5.1.1. Cellular factors

5.1.1.1. Morphology of haemocytes

Investigations into the haemocytes of *F. indicus* revealed three types of cells, under both light and electron microscopy and were classified into hyalinocytes, small granule haemocytes and large granule haemocytes.

On Wright's staining of haemolymph smears, three forms of cells were observed. One category of cells was small, had very little amount of cytoplasm and was devoid of cytoplasmic granules. These cells were termed hyalinocytes. The next category of haemocytes was larger, contained abundant cytoplasm with small, distinct, basophilic granules and was named small granule haemocytes. The last type of haemocyte was larger than the other two types of haemocytes. The distinguishing feature of these cells was the presence of large, highly acidophilic granules seen in the abundant cytoplasm, and was termed large granule haemocyte.

Electron microscopy also showed the presence of three types of haemocytes in the haemolymph. The first category of cells had large nucleo-cytoplasmic ratio and contained no cytoplasmic granules. This category was called the hyalinocytes. The second type had large amounts of cytoplasm with well developed rough endoplasmic reticulum and number of mitochondria and many small electron dense cytoplasmic granules. Thus, they were termed small granule haemocytes. The last type of cells contained very large electron dense granules that were scattered in the cytoplasm. These were large granule haemocytes. These cells also contained rough endoplamic reticulum and mitochondria. Intermediate stages of the three classes of haemocytes were encountered in the investigation. The presence of intermediate stages implies that absolute categorization into hyalinocyte, small granule haemocyte and large granule haemocyte is arbitrary and these are different stages during cell

development. Earlier studies have indicated that hyaline cells develop into small granule haemocytes, and small granule haemocytes progress into large granule haemocytes (Cuénot, 1891; Bruntz, 1907; Arvy, 1952; Wood and Visentin, 1967; Benjamin and James, 1987).

The presence and absence of cytoplasmic granules is the main feature in all the classification schemes of haemocytes. Although the terms vary, almost all crustaceans seem to have haemocytes with and without granules. Based on the staining characteristics, cytoplasmic granules when present seem to be of two types; either acidophilic or basophilic. Regarding the size of the cytoplasmic granules, small and large granules have been noticed. The observations in the present study clearly indicate that *F. indicus* has agranular cells called hyalinocytes; haemocytes termed small granule haemocytes containing basophilic granules that are small and spherical, and large granule haemocytes containing eosinophilic granules that are very large, irregular in shape and numerous in numbers. The well developed rough endoplasmic reticulum and polyribosomes along with the large number of mitochondria seen in the small granule haemocytes indicate that they are active cells with high metabolic and synthetic activity. According to Benjamin and James (1987) the hyalinocytes are undifferentiated cells, the small granule haemocytes are highly active forms and large granule haemocyte is the end point of the maturation process of a cell. The present observations also indicate a possibility similar to the above observation.

This description is similar to that of Laxmilatha and Laxminarayana (2004) on the haemocytes of *F. indicus,* where they termed the cells as agranulocyte, semi-dense granulocyte and dense granulocyte. The fourth type of cell, the cyanocyte, assumed to be seen in females with developing ovary was not observed in the present study as juveniles alone were chosen and sex differences were not considered.

Penaeids like *S. ingentis, P. japonicus, P. monodon, P. penicillatus, F. paulensis, F. californiensis, L. vannamei* and *L. stylirostris* also showed three types of haemocytes similar to that of *F. indicus* (Martin and Graves, 1985; Tsing *et al.*, 1989; Gaargioni and Barracco, 1998; Yip and Wong, 2002; Vargas-

Albores *et al.*, 2005). In addition to these three cells, *P. monodon* possessed large granule haemocytes with large inclusions of a granular content. Other crustaceans also have different types of cells which differ in the presence and/or absence of granules. The crab, *P. fluviatilis*, the Dungeness crab, *C. magister*, and the Blue crab, *C. sapidus* displayed agranular cells, semi or small granular cells and granulocytes or large granule haemocytes (Bodammer, 1978; Mix and Sparks, 1980; Yavuzcan-Yildiz and Atar, 2002). The lobster, *H. americanus*, the crayfish, *Procambarus* spp., *Orconectus* spp. and *P. clarki*, and the horse-shoe crab, *L. polyphemus* have agranular and granular cells with basophilic or eosinophilic granules, small or large in size, similar to that of *F. indicus* (Hearing and Vernick, 1967; Cornick and Stewart, 1978; Sternshein and Burton, 1980; Copeland and Levin, 1985; Lanz *et al.*, 1993). Unlike in *F. indicus*, the hyalinocytes of the palaemonids *M. rosenbergii* and *M. acanthurus* contain granules in the cytoplasm (Gaargioni and Barracco, 1998).

5.1.1.2. Cytochemical studies

Cytochemical observations in *F. indicus* revealed the presence of carbohydrates, lipids, the enzymes prophenoloxidase, peroxidase and acid phosphatase in the haemocytes. Not all haemocytes were positive to the various cytochemical tests, implying that the haemocytes of *F. indicus* vary in their physiological role.

Carbohydrate positive granules were observed in less number of haemocytes of *F. indicus*. The nature of the haemocytes positive to PAS staining and the nature of the carbohydrate could not be ascertained. Carbohydrates are found in different forms in various cells as mucopolysaccharides, glycoproteins, and energy reserves or as degenerative material from phagocytosed microorganisms (Dall, 1964; Hose *et al.*, 1987). Carbohydrate in the form of the energy reserve, glycogen, has been detected in many crustaceans as in *A. salina*, *P. marmoratus* and *O. virilis* and is usually seen in the cytoplasm as deposits (Lockhead and Lockhead, 1941; Arvy, 1952; Wood and Visentin, 1967). In *C. maenas*, glycogen was seen as cytoplasmic deposits in hyaline cells and in

between the granules of granulocytes (Johnston *et al.*, 1973). But, William and Lutz (1975) observed glycogen containing granules and non-glycogen polysaccharide granules in *C. maenas*. In *E. sinensis*, the granules of the haemocytes contained the structural polysaccharide, chitin (Bauchau *et al.*, 1975). Cytoplasmic granules may also contain carbohydrates in the form of mucopolysaccharides, which may be neutral or acidic in nature (Dall, 1964). In *S. ingntis*, haemocytes especially the agranular haemocytes contained carbohydrates in the form of glycoproteins (Hose *et al.*, 1987).

The small number of carbohydrate positive cells in *F. indicus* implies that these play a minor role in carbohydrate metabolism and transport. Another possibility is that these cells may generate molecules, like lectins, which are carbohydrate in nature, only on stimulation. Further studies into the nature of the carbohydrates and the type of haemocytes containing them will help in understanding the role of carbohydrates in the metabolism of *F. indicus*.

Two types of staining reactions were noticed in *F. indicus* haemocytes, on Sudan Black B staining. In some cells, dark stained areas along the periphery was observed while in others, distinct, dark granules were seen in the cytoplasm. According to Bauchau (1981) although lipids are seen in all haemocytes, they accumulate in the granules of lipoprotein cells alone. Lipid moieties are also linked to glycoprotiens, where they are seen as deposits as was observed in *S. ingentis*, in which some of the small granule haemocytes contained lipoglycoprotein deposits (Hose *et al.*, 1987). In the present study, the cells containing dark granules may be assumed to be lipoprotein cells. The other cells displaying dark staining along the periphery seem to be lipoglycoprotein in nature as cited by Hose *et al.* (1987). This indicates the abundant presence of lipids in the haemocytes of *F. indicus*.

In *F. indicus*, majority of the haemocytes were positive for prophenoloxidase enzyme activity. Of these, some gave intense activity and from their morphology, these cells appeared to be large granule haemocytes. The enzyme was seen diffused in the cytoplasm of the haemocytes of *F. indicus*. In *S. ingentis*, prophenoloxidase activity was observed in large and small granule

haemocytes. All the granular haemocytes had prophenoloxidase activity at the D-moult stage. At intermoult stage, the activity decreased (Hose *et al.*, 1987). Where as, in *P. japonicus,* phenoloxidase activity was found only in large granule haemocytes and it was seen dispersed in the cytosol (Tsing *et al.*, 1989). It is believed that phenoloxidase activity is restricted to the granulocytes, as it was absent in the hyalinocytes of *C. maneas, S. ingentis*, and *P. japonicus* (Söderhäl and Smith, 1983; Hose *et al.*, 1987; Tsing *et al.*, 1989). This may be true in the case of *F. indicus* also, as most of the cells in the sample gave a positive reaction. The morphology of such positive haemocytes in *F. indicus* indicates that these are large and small granule haemocytes. Of these, the large granule haemocytes gave intense reactions and the small granule haemocytes showed less intense activity.

Acid phosphatase enzyme activity was confined to the cytoplasmic granules of *F. indicus*. Not all the cells of *F. indicus* gave a positive reaction for the enzyme. Acid phosphatase is a lysosomal enzyme and is involved in the degradation, hydrolysis of acylglycerol and hydrolysis of peptides bearing free amino acids and is generally found in cells capable of phagocytosis (Chu, 2000). This enzyme is present in the granules of haemocytes, particularly in the small granule haemocytes. In *S. ingentis* and *P. japonicus,* acid phosphatase activity was observed mainly in the granules of small granule haemocytes. The occurrence of this lysosomal enzyme in these cells implies the phagocytic nature of these cells (Brehèlin and Arcier, 1985; Hose *et al.*, 1987; Tsing *et al.*, 1989). Phenoloxidase has been observed in the granules of large granule haemocytes of *S. ingentis*, but the activity was less than the small granule haemocytes (Tsing *et al.*, 1989). The granulocytes of *F. paulensis* have vesicles containing acid phosphatase (Gargioni and Barracco, 1999). Since, in the present study, the acid phosphatase activity was confined to the granules of haemocytes, it can be assumed that the granulocytes of *F. indicus* are the cells involved in phagocytosis. Cytochemical investigations on this enzyme in crustaceans are limited.

Majority of the haemocytes of *F. indicus* exhibited peroxidase activity in the granules. Peroxidase enzyme is involved in oxygen metabolism in both vertebrates and invertebrates. During oxygen metabolism, a number of toxic reactive oxygen radicals are produced. They are superoxide ion radical (O_2^-), hydrogen peroxide (H_2O_2), and the most reactive species, hydroxyl radical (OH^-). Superoxide anion splits into hydrogen peroxide and oxygen in the presence of superoxide dismutase. The hydrogen peroxide is reduced by the enzyme peroxidase in the presence of a reducing agent to form water. Catalase also helps to convert hydrogen peroxide into water. Thus peroxidase, catalase and superoxide dismutases are anti-oxidative enzymes that take up reactive oxygen radicals. At times of microbial interference or other such stages of risk to the organism, these toxic substances are directed towards the intruders. Hydrogen peroxide is having anti-bacterial activity. The peroxidase enzyme oxidizes substrates in the presence of hydrogen peroxide, thus producing highly reactive intermediates. Oxidation of phenol to quinone is an example (Holmbald and Söderhäl, 1999). Peroxidase activity has been demonstrated in the ridge-back prawn, *S. ingentis* (Hose *et al.*, 1987). The occurrence of peroxidase activity in the haemocytes of penaeid species is an important observation. This indicates that the haemocytes play a major role in the immune reactions of shrimp. Hence investigations into the dynamics of haemocyte activity will yield valuable clues useful for diagnostic and immunological evaluations of penaeids under threats of disease out breaks.

5.1.1.3. Total haemocyte count (THC) and differential haemocyte count (DHC)

In invertebrates, haemocytes have many important functions to play, which may be physiological or pathological. Carbohydrate transport, coagulation, phagocytosis and encapsulation are some of these functions (Ravindranath, 1980; Bauchau, 1981). The total haemocyte count or the total number of cells present in one millilitre of haemolymph indicates the number of cells available for these mechanisms. The cellular profile or the differential haemocyte count gives a

picture of the different types of cells possessed by the animal which take part in the various mechanisms of the animal, of which, defense functions are crucial to the animal. Stress conditions modulate total haemocyte and differential haemocyte count. The fluctuation in total haemoctye count is due to the redistribution of haemocytes between a circulating pool of cells and a non circulating pool in which cells are probably adhered to the haemal linings of the open circulation system (Noga, 2000). The agranular and granular cells have specific functions and respond differently to stress. These parameters provide a way of assessing the physiological state of the animal (Martin and Graves, 1985). The values also range among the different species. This may be due to the different environments in which these animals live in.

In the present study, the total haemocyte count of apparently normal *F. indicus* ranged from 1315 to 2431 $\times 10^4$ cells/ml of haemolymph, with a mean value of 1978.77± 4.28$\times 10^4$ cells/ml of haemolymph. The information on the total haemocyte count and the differential haemocyte count of the haemocytes of *F. indicus* are scanty. However, in many other species of prawns varying numbers of total haemocyte count has been reported. According to Chang *et al.* (1997) the mean total haemocyte count of penaeid shrimp range from 20 to 40 $\times 10^6$ cells/ml. Varying values have been reported in *P. monodon* ($14\pm6\times10^6$ cells/ml), *P. penicillatus*, *P. japonicus* (4-5 $\times10^6$ cells/ml), *F. californiensis* ($4.0\pm1.6\times10^6$cells/ml), *L. vannamei* (9.35±2.1$\times 10^6$ cells/ml) and *L. stylirostris* ($14.1\pm5.3\times10^6$ cells/ml) (Rengpipat *et al.*, 2000; Yip and Wong, 2002; Vargas-Albores *et al.*, 2005). The total count recorded in the crabs, *C. maenas* (26.5×10^6 cells/ml) and *C. sapidus* (5.8×10^7 cells/ml) was greater than the values observed in shrimps (Smith and Söderhäll, 1983; La Peyre and Chu, 1990). However lower total haemocyte counts were observed in other crustaceans like *H. americanus*, *S. ingentis*, *F. californiensis* and *L. oceanica* (Cornick and Stewart, 1978; Martin and Graves, 1985; Benjamin and James, 1987).

The values of total haemocyte count of crustaceans range widely, between species and within species. This is mainly because of the wide range of environments they dwell in and also due to the physiological states of these

animals (Yeager and Tauber, 1935; Noga, 2000). Different cell counts are encountered in the same animal by different authors. For example, contradictory to the value obtained by Hardy (1892), *A. astacus* had a greater cell count in the study of Söderhäll and Smith (1983). The freshwater crab, *Potamon fluviatilis* had a total haemocyte count of $10.3\pm11.10\times10^5$ cells /ml of haemolymph but showed high intra specific variation in the total count (Yavuzcan-Yildiz and Atar, 2002). Total counts are seen to vary during moult cycles (Stewart *et al.*, 1967). It is essential to arrive at the baseline data for haemocyte counts at different physiological stages and stress conditions. This will help to predict the susceptibility of the species under investigation to various bacterial and viral diseases.

In *F. indicus*, the present study clearly showed that the small granule haemocytes form the majority of haemocytes, followed by the hyalinocytes, with large granule haemocytes forming a minor group. In the differential haemcyte count (DHC) of apparently normal animals, the small granule haemocytes varied from 54% to 77% and their mean value was 67.77 ± 6.35%. The hyalinocytes ranged from 20 to 28% and gave a mean value of 22.92 ± 2.82%. Large granule haemocytes varied from 3 to 11% with a mean value of 6.44 ± 2.8%. In many other crustaceans also, the small granule haemocytes, known by different names, are more in number compared to the other types of cells (Hearing and Vernick, 1967; Cornick and Stewart, 1978; Mix and Sparks, 1980; Martin and Graves, 1985, Yavuzcan Yildiz and Atar, 2002). But, in *L. oceanica* (Benjamin and James, 1987), the small granule haemocytes, were less, compared to hyalinocytes and granulocytes. In this species, the granulocytes were more, followed by the hyalinocytes. Some times the percentage of hyalinocytes to large granule haemocytes ratio also varies with species. In *F. indicus*, the hyalinocytes were more than the large granule haemocytes. The large granule haemocytes showed the lowest percentage. A similar result was seen in *S. ingentis*, where, the hyalinocytes were more than the large granule haemocytes. However, in *F. californiensis*, the large granule haemocytes were more than the hyalinocytes (Martin and Graves, 1985). In *H. americanus*, the percentage of hyalinocytes and

granulocytes were the same. This was same in *C. sapidus* and crayfish also. In *C. magister*, the hyalinocytes and eosinophils (large granule haemocytes) both showed wide ranges (Hearing and Vernick, 1967; La Peyre and Chu, 1990; Sternshein and Burton, 1980; Mix and Sparks, 1990).

5.1.1.4. Phagocytosis

In the present study, mean percentage of phagocytosis recorded in apparently healthy *F. indicus* juveniles was 53.15 ± 8.87 %. Half the haemocyte population is capable of phagocytosis, as estimated by particulate phagocytic stimulant, baker's yeast. Phagocytosis is among the most primitive immunodefense mechanisms (Cooper, 1995). Phagocytosis of microbial agents and non-self materials represent an important defense mechanism in vertebrates and invertebrates and includes the process of recognition, adherence, ingestion, destruction and disposal. Recognition of foreign cells or damaged tissue is achieved by plasma and haemocyte membrane-associated lectins, which facilitate binding of the haemocytes and non-self material, resulting in efficient phagocytosis of the entity (Chu, 1988; Adema *et al.*, 1993).

Ingestion of foreign particles is based on three different phenomena: 1) particle adhering to the cell filopodia is taken into the ectoplasm of the filopodia and then enclosed in a phagosome (Bang, 1971); (2) an invagination forms on the surface of the cell and the particle is enclosed into a vesicle (Cheng and Rodrick, 1975); (3) the particle is taken into a funnel-like pseudopodium produced by granulocytes and hyalinocytes (Renwrantz *et al.*, 1979). Destruction or intracellular killing of the ingested particle is either realized by lysosomal enzymes and /or formation of reactive oxygen intermediates (ROIs).

Earlier studies have reported low phagocytic values in crustaceans (Smith and Ratcliffe, 1978; La Peyre and Chu, 1990). However, in *S. ingentis* 63% of the total cells were capable of phagocytosis. Of these cells, 31.6% of small granule haemocytes and only 6.2% of large granule haemocytes exhibited phagocytosis. Their percentages, especially of the small granule haemocytes increaseD with time (Hose and Martin, 1989). In *P. japonicus,* the phagocytic

activity of the granulocyte was 77.7 %. In this species too, it was thought that phagocytic activity was confined to the granular cells, which have, lysosomal enzymes stored in their cytoplasmic granules (Hose *et al.*, 1987; Tsing *et al.*, 1989). But, in *P. japonicus, P. monodon, A. astacus* and *P. leniusculus*, both hyaline and granular cells are capable of phagocytosis (Smith and Söderhäll, 1983; Kondo *et al.*, 1992; Ekpanithanpong *et al.*, 1999). In the present observations, 53% of the cells were found to be phagocytic. The study could not differentiate the different cell types involved in phagocytosis. The cytochemical localization of acid phosphatase, peroxidase and the phenolpxidase activities located in the granules implies that the granulocytes play important role in phagocytosis. The strong peroxidase activity was further confirmed by the superoxide formation.

5.1.1.4.1. Oxygen radical formation

In *F. indicus* haemocytes, strong NBT reduction was seen with the production of large numbers of formazan granules in the phagocytosing cells. This indicate that the phagocytic activity of the haemocytes involve the production of reactive oxygen intermediates which play an important role in foreign body elimination. Peroxidase activity and superoxide production play important roles in defense mechanisms.

Literature on reactive oxygen radicals in crustaceans is limited. Several studies have been conducted in molluscs (Dikeboom *et al.*, 1985; Bachère *et al.*, 1991; Pipe, 1992). In crustaceans, superoxide anion production has been reported in *C. maenas, P. monodon, P. japonicus* and *L. vannamei* (Bell and Smith, 1993; Song and Hsieh, 1994; Bachère *et al.*, 1995; Gullian *et al.*, 2004). In the present study, *F. indicus* haemocytes showed strong phagocytic activity and a good profile of phagocytic enzymes like acid phosphatase and peroxidase. The haemocytes also elicited a very strong superoxide production activity during the phagocytosis. All these reactions indicated that the haemocytes are active phagocytes capable of resisting microbial invasion.

5.1.2. Humoral factors

Humoral responses of invertebrates are actually special cases of cellular responses, in which, secretion, fragmentation, or bio-chemical alteration of the haemocytes confer bacteriostatic, lytic, or other properties of body fluid (Stauber, 1961).

Humoral factors consist of various protein molecules whose origin is believed to be cellular, which associate themselves with intracellular or extracellular killing. The phenoloxidase and the pro-phenoloxidase system, various non-specific agglutinins or lectins and various microbicidal lytic enzymes are some of the known humoral factors. The molecules are stored in the cytoplasmic granules and are released extracellularly during immune response. Thus, the serum or the fluid part of the haemolymph is a mixture of various protein fractions involved in the immune system. Since it is a ready and easy source of factors involved in humoral immune mechanisms, serum can be used to monitor and analyze the health and physiological status of cultured animals (Parazzolo and Barracco, 1997).

5.1.2.1. Serum Protein profile

According to Florklin (1960), total protein in decapods vary from 0.7 to 8.8 g/100 ml. The total serum protein concentration of *F. indicus* recorded in the present study fall in this range. The mean protein value obtained was a mean of 55.55±12.57 mg/ml, showing a minimum of 34.5 mg/ml and a maximum of 72 mg/ml. Jayasree and Selvam (2000) observed a protein concentration of 108.91 mg/ml in *F. indicus*. This higher protein value could be due to the larger size of the animals used in their study, compared to those used in the present study. Increase in protein concentration with size has been reported earlier (Laxmilatha, 1991).

Interspecies and intraspecies variation in total protein concentration can be seen in many crustaceans. Values comparable to *F. indicus* were seen in crustaceans such as *P. esculentus* where the serum protein concentration was of

82 mg/ml and *C. maenas* which had a mean total protein concentration of 60 mg/ml (Webb, 1940). High variations are observed in the total protein values of many crustaceans such as C. *sapidus*, *C. magister, C. iroratus*, *L. emargirulus*, *H. americanus* and *L. polyphemus* (Leone, 1953). In the isopods, *Oiniscus asellus*, *Porcellio scaber* and *Cylisticus convexus* the protein values of 2.9, 3.07 and 3.72 g/100 ml were seen (Gondko *et al.*, 1984). The protein concentration in *F. paulensis* was 146±30 mg/ml or 14.6 g/100ml (Perazzolo *et al.*, 2002). It was much greater than that of *F. indicus*. Variations in the serum protein value between individuals and within individuals are common due to the varying environments they reside in. Variations even during the day have been reported in *S. serrata* due to periodic sequestering of proteins from different tissues at different times of the day (Subhashini and Ravindranath, 1980).

Electophoresis of body fluids reveal the different protein fractions, which differ qualitatively and quantitatively from species to species (Stewart *et al.*, 1966; Cuzon and Ceccaldi, 1972; Martin *et al*, 1977; Alikhan and Aktar, 1980; Prathibha, 1984). SDS-PAGE was conducted on the serum of healthy *F. indicus* and revealed 14-16 polypeptide bands. One of the bands showed high intensity and had a molecular weight between 70 to 80 kDa. Other bands of moderate intensity were of molecular weights 56, 44, 41, 40, 38, 24 and 20 kDa. Feeble fractions were also encountered, of which, two to three fractions weighed above 100kDa. The nature of these polypeptide bands was not confirmed. Laxmilatha (1991) reported a maximum of 16 to a minimum of 10 protein bands on conducting SDS-PAGE on the haemolymph of *F. indicus* (Laxmilatha, 1991). Of these, four major protein fractions along with other minor fractions were obtained. The major polypeptides were slow and fast haemocyanin, heteroagglutinin and fibrinogen. The two hemocyanin fractions formed 60-80% of the total protein content of the body. The fast haemocyanin fraction appeared as a conspicuous broad band. The heteroagglutinin, slow haemocyanin, and the fibrinogen fractions were of greater molecular weight and were seen above the fast hamocyanin. In the present study, the broad band is assumed to be haemocyanin as in the study of Laxmilatha (1991). The minor polypeptide bands

were in the range of 56 kDa to 20 kDa. Nevertheless, these were species specific as the pattern remained fixed in the animals studied. In the present study, serum was used instead of haemolymph. When clotting occurs, fibrinogen is lost and the serum formed is devoid of this fraction. This may be the reason for the difference in the number of protein bands observed in the present study. Similar differences in the electophoretic pattern were seen in *H. americanus*. Stewart *et al.* (1966) conducted electrophoretic and sedimentation analysis on the plasma and serum of the lobster, and found that the plasma of the lobster has six components while the serum has only five.

5.1.2.2. Haemagglutination

The haemagglutinins of *F. indicus* showed high agglutinating activity. The haemagglutinating titer, expressed as the reciprocal of the maximum dilution giving positive agglutination, varied from 16 to 64. The mean value obtained for *F. indicus* was 33.77 ± 18.66. High variations in the agglutination titers were seen in the rock lobsters, *J. novaehollandiae*, *J. edwardsii* and *J. verraexi* (Imai *et al.*, 1994). The haemoplymph of *J. novaehollandiae* expressed inconsistent agglutinating activity against human RBC and the titers ranged from 4 to 1024. The serum of *P. japonicus* agglutinated chicken RBC only after incubation at 6°C for 16 hours. The activity was low, but it showed greater activities with human and horse RBC and did not require temperature modifications (Ratanpo and Chulavatnatol, 1990). The haemagglutinating activity in *F. indicus* against chicken RBC did not depend on temperature or time. Agglutination was complete within 1 hour and incubation at 4°C did not increase the titer.

Many workers separated the agglutinating fractions from the haemolymph by various methods and tested the fractions. The major lectin in *J. novaehollandiae* called JN-2 was a glycoprotein and on SDS-PAGE, dissociated into subunits of molecular weights 85, 81 and 63 kDa. In *P. monodon* SDS-PAGE expressed two sub-units of molecular weight 42 kDa and 27 kDa and a major band of molecular weight 33kDa (Muramoto *et al.*, 1995). Though no particular fraction from the protein of *F. indicus* could be isolated with

agglutinating activity, bands of 41, 38, and 24 kDa were found in the haemolymph which may be associated with haemagglutination activity. This needs further investigation.

5.1.2.3. Enzyme profile

5.1.2.3.1. Phenoloxidase

The mean phenoloxidase activity of serum of *F. indicus* was 1.1640 ± 0.59 enzyme units and ranged from 0.667 to 2.57 enzyme units. Comparable activities were seen in the edible crab, *C. pagurus* that measured 2.89 ± 0.18 units (Vogan and Rowley, 2002). The phenoloxidase activity in *P. monodon* was 33.7 units /min /mg protein (Rengpipat *et al.*, 2000). The activity increased during captivity. Phenoloxidase is the terminal enzyme in the prophenoloxidase system. Prophenoloxidase, its zymogen, is activated to phenoloxidase by endogenous sereine proteases, by external compounds like laminarin, β-glucans, lipopoysachharides and anionic detergents. The prophenoloxidase system is a complement-like enzyme cascade and results in the production and deposition of melanin during encapsulation process (Söderhäll, 1981). Quinones formed during this process are anti-microbial. Phenoloxidase is also known to produce opsonins and cell-adhesion molecules (Söderhäll, 1981; Söderhäll and Cerenius, 1992) that recognize non-self molecules from self.

5.1.2.3.2. Acid and Alkaline phophatase

The concentration of acid phosphatase in the serum of apparently healthy *F. indicus* was in the range of 26.12 to 42.9 KA units. The recorded mean value was 33.59 ± 7.5 KA units. The serum alkaline phosphatase level of apparently healthy shrimp was in the range of 2.055 and 3.04 KA units, with a mean of 2.309 ± 0.318 KA units. Although their presence has been confirmed in crustaceans (Hose *et al.*, 1987; Lanz *et al.*, 1993; Song and Hsieh, 1994; Sung *et al.*, 2000) quantitative estimates of these two enzymes are few. Acid phosphatase in the serum of the oyster, *C. virginica* was 0.67±0.35 milliunits (mU)/ mg protein. The enzyme activity of the haemocytes was much more than the serum

activity being 46.79±5.24 mU/mg protein (Cheng and Rodrick, 1975; Cheng and Downs, 1988). The acid phosphatase activity recorded in *F. indicus* in the present study and those in the oyster, *C. virginica*, although are similar, cannot be compared as they belong to different phyla. In the present study, *F. indicus* showed a mean alkaline phosphatase activity of 2.3 ± 0.32 KA units and was comparable to the alkaline phosphatase activity of the serum of *P. marginatus* and *M. rosenbergii* which were 4.6 and 7.8 KA units, respectively (Balazs *et al.*, 1973).

5.2. Immunomodulation

5.2.1. Exposure to biological agent, *V. parahaemolyticus*

5.2.1.1. Cellular factors

The total haemocyte count has been correlated to disease occurrence in crustaceans. Decrease in the total count or haemocytopenia has been recorded in *C. maenas* during experimental and natural infections and also in *P. monodon* (Smith and Ratcliffe, 1980; Bang, 1983; Hauton *et al.*, 1997; Supamattaya *et al.*, 2000). In *F. indicus,* there was a significant reduction also in the number of circulating haemocytes in the initial stages after injection of bacteria, which stabilized only after one week. The initial reduction in the haemocyte number might have been due to the mobilization of haemocytes from circulation and selective adherence of the haemocytes to haemal sinuses for effective clearance of bacteria. The haemocyte count stabilized only after 1 week post injection. The prolonged decline implies that the cells were engaged in the host's defense mechanisms (Smith and Ratcliffe, 1980; Bang, 1983; Ratcliffe and Gagen, 1976; 1977). In *H. americanus* injected with bacteria, a reduction in the haemocyte number associated with clearance of injected material has been observed (Cornick and Stewart, 1968). *L.polyphemus*, injected with Gram negative bacteria (Bang, 1983) and the wax moth, *G. mellonella* on injecting bacteria (Ratcliffe and Gagen, 1976) also showed reduced number of circulating haemocyte in the initial stages.

Infections are not always accompanied by a decrease in cell count. In crayfish, the parasite *Psorospernium hacheli* caused an increase in the total haemocyte count (Söderhäll and Cerenius, 1992). In some crustaceans, like the crab, *C. pagrus* bacterial infections have not caused changes in the dynamics of the total cell count (Vogan and Rowley, 2002). However, in *F. indicus*, the bacterial introduction resulted in significant fluctuations in the total counts of the haemocytes, which were time dependent. The results indicate the possibility of using total haemocyte count as an indicator of bacterial infections.

The bacteria injected animals showed an initial increase in the percentage of hyalinocytes, which decreased in time. In contrast, the percentage of small granule haemocytes of these animals were less initially, but increased afterwards. According to many workers, the small granule haemocytes are the primary cells involved in phagocytosis (Smith and Ratcliffe, 1980; Benjamin and James, 1987; Tsing *et al.*, 1989; Hose and martin, 1989; La Peyre and Chu, 1990). Present study agrees with the study of Smith and Ratcliffe (1980) in which, a reduction in the phagocytic cells in *C. maenas* after bacterial injection was observed. The decrease in the percentage of small granule haemocytes at 24 hours could be attributed to their participation in the defense reactions such as phagocytosis and encapsulation reaction. This requires mobilization of circulating small granule haemocytes from the haemolymph towards specific areas of pathogen attack. The bacterial infection requires the presence of more phagocytic cells and these cells are formed from hyalinocytes. Hence, a reduction in the hyalinocyte number after the initial hours was seen due to the conversion of hyalinocytes into small granule haemocytes, thus leading to an increase in their percentage in circulation.

The percentage of large granule haemocytes in test animals decreased with time. At 3 hours there was a significant reduction of these cells in the test. Small granule haemocytes are considered to be the precursors of large granule haemocytes (Bodammer, 1978). As the bacterial infections demands more number of the phagocytic, small granule haemocytes, their conversion to large granule haemocytes is delayed. This might have caused the relative decrease in the percentage of large granule haemocytes (Smith and Ratcliffe, 1980).

The phagocytic activity of the haemocytes was not significant at any of the time intervals. *V. parahaemoclyticus* is an opportunistic pathogen and becomes pathogenic at times of stress. In the present study, *in vitro* phagocytic assay was studied using yeast cells. As the phagocytic cells were already involved in the phagocytosis of the pathogenic bacteria injected, their affinity towards the yeast cells was low. This may be the reason for the non-significant result obtained in the present study.

5.2.1.2. Humoral factors

Experimental infection in *H. americanus* (Stewart *et al.*, 1969) resulted in a decline in the protein concentration. In *F. indicus*, the total serum protein concentration decreased in the initial 30 minutes of post injection. The initial drop in the protein level might have been due to the introduction of pathogen into the body of the animal. At 3 hours and 24 hours the protein level of the serum increased, which was followed by a decrease. This rise in the protein levels might be associated with the release of other humoral defense factors involved in clearance of bacteria. Haemagglutinins increased in *F. indicus* after the initial period. Concentration of haemagglutinins vary in the presence of pathogens (Norton *et al.*, 2000). The phenoloxidase activity was low at 30 minutes, but increased significantly at 3 hours and afterwards. Increase in the phenoloxidase activity was also seen in *P. chinensis* and *C. maenas* (Barracco and Söderhäll, 1996; Li *et al.*, 1998).This implies that the presence of *V. parahaemolyticus* increased the defense reactions of the shrimp in the initial stages as a measure to restrict the advance of the pathogenic cells. Like wise, the increase in phenoloxidase activity was significantly high even after two weeks. Presence of pathogens at sub-lethal levels may have a stimulating effect on certain humoral factors.

A decrease in the serum acid phosphatase concentration of the test animals was observed at 3 hours, compared to control. At 24 h, the enzyme activity in the test animals was significantly greater than the control animals. Acid phosphatase is a hydrolytic enzyme of lysosomal origin and is involved in

intracellular and extracellular killing. It is an enzyme that catalyzes the hydrolysis of orthophosphoric acid esters at optimal pH below 7. Acid phosphatase reaches the serum through degranulation. This natural process is enhanced by the presence of bacteria. Release mechanisms of the phosphatase are fast, occurring after 30 minutes of bacterial exposure (Mohandas *et al.*, 1985). The significant rise in the enzyme activity at 24 hours after bacterial injection indicates the involvement of the enzyme in bacterial clearance in *F. indicus*. The subsequent decrease during the remaining period implies that the clearance ability of *F. indicus* is rapid. Acid phosphatase is also known to increase in *M. rosenbergii* (Ramalingam and Ramarani, 2004) on exposure to endotoxin of *Psuedomonas aeruginosa* (MTCC 1988).

Alkaline phosphatase activity decreased in the serum of bacteria injected *F. indicus*. The concentrations were low during the entire experimental period. This was contradictory to the observations of Ramalingam and Ramarani (2004) who observed an increase in the alkaline phosphatase activity in *M. rosenbergii* challenged with the endotoxin of *P. aeruginosa* (MTCC 1988). Alkaline phosphatases hydrolyze orthophosphoric acid esters at pH above 7, i.e., at an optimal pH of 9.6. Although known as a lysosomal enzyme, the true relevance of this enzyme against bacteria is still vague. Even though the optimum pH of this enzyme is greater than the physiologic pH, negatively charged groups found in bacterial endotoxin activates the enzyme at the physiologic pH level. In the present study, it may be assumed that the sub-lethal concentration of *V. parahaemolyticus* could not provide the sufficient negatively charged groups that promote enzyme activity in *F. indicus*. This may be the reason for low alkaline phosphatase activity throughout the experiment.

5.2.1.3. Histological changes

During the initial hours, slight changes were seen in the gills, heart and hepatopancreas. The haemal sinuses of the secondary gill filaments were engorged due to haemolymph accumulation. Regions in the heart also showed areas of engorgement with loss of striations at certain parts. The haemal sinuses

between the hepatic tubules also showed enlargement. There was infiltration of haemocytes in the antennal gland tubules. After 24 hours, the haemal sinuses of the hepatopancreas still showed dilation. After 48 hours, haemal sinuses of gills and the antennal gland tubules exhibited exudation. After this, no prominent change in any of the tissues were detected. In *F. indicus* and *P. monodon*, destruction of the gills, hepatopancreas and haemopoetic tissue resulted from lethal concentrations of *V. parahaemolyticus*. The bacterium upon entry into the host, induce systemic infection and interfere with normal activities of the hepatopancreas and causes myocardial necrosis and destruction of the haematopoetic tissue (Shahul Hameed, 1989). *P. monodon* infected with *V. alginolyticus* also showed changes in the gills, antennal gland and hepatopancreas (Tressprateep *et al.*, 1990). The changes in *F. indicus* in the present study were confined to the early period of the infection. Scince the concentration of the bacteria used in the study was sub-lethal, it would have been easily cleared from the system.

5.2.2. Exposure to pollutants

5.2.2.1. Nuvan

5.2.2.1.1. Cellular factors

In *C. crangon*, concentrations as low as 5 % of dredge spoil, containing various substances such as insecticides, pesticides, heavy metals etc., decreased the total haemocyte count (Smith *et al.*, 1995). Low concentrations of nuvan also caused a reduction in the total haemocyte count of *F. indicus*. Decrease in the cell number is caused by the adherence of cells within various vascular channels as in the case of microbial infection (Bang, 1983). It may also be due to cell lysis. As the concentration increased, there was an increase in total haemocyte count. The highest total haemocyte count was seen at 0.08 ppm. Haemocytes have glycogen reserves that provide energy to the animal (Lockhead and Lockhead, 1942; Arvy, 1952; Wood and Visentin, 1967). Increase in total circulating cells may be to increase the availability of energy to the stressed animal. Another possibility is that the ability to selectively adhere to key areas is lost, as the

organophosphates interfere with acetylcholine esterase activity and integrity of cell membrane (Lignot *et al.*, 1997). As a result, the animals may be susceptible to infection due to presence of pesticides.

There was significant variation in the differential count of the animals on exposure to nuvan. At 0.05 ppm nuvan, percentage hyalinocytes increased while the percentage of small granule haemocytes decreased. Hyalinocytes are believed to be the precursors of small granule haemocytes (Bodammer, 1978). An increase in the hyalinocytes and a decrease in the small granule haemocytes at 0.05 ppm concentration imply that the conversion of hyalinocytes into small granule haemocytes is hindered at this concentration. There was a significant reduction in the phagocytic activity at 0.05 ppm nuvan. Since small granule haemocytes are the main participants in phagocytosis, the phagocytic activity may be compromised at this concentration (Benjamin and James, 1987; Hose and Martin, 1989; Tsing *et al*, 1989). Thus, it can be imagined that in *F. indicus*, hyalinocytes donot have a major role in phagocytosis and that phagocytosis is carried out mainly by the small-granule haemocytes. The sublethal concentration of 0.05 ppm nuvan may have adversely affected the conversion of hyalinocytes to small granule haemocytes and thus may have affected the phagocytic ability and resulted in the compromise of defense mechanism.

Interestingly the large granule haemocytes showed significant decrease at 0.02 as well as 0.08 ppm pesticide, while the other cells did not show any significant differences in the percentage, at these concentrations. The individual haemocyte percentages are the relative estimation of the total haemocyte count. In low concentration of pesticide, total haemocyte count showed significant decrease, where as, at higher concentration total haemocyte count showed a significant rise. Thus, it is seen that the initial decrease of the large granule haemocyte was an absolute decrease in their number. But the decrease noticed in the higher concentration was due to a relative fall, because of the increase in the number of other cells. The hyalinocytes formed may not be maturing ito small and large granule haemocytes, resulting in a reduced percentage of small and large granule haemocytes and a high percentage of hyalinocytes. It may be

possible that mobilization of cells from tissues might have occurred at higher concentration of pesticide, which resulted in the increase in number of haemocytes.

Dichlorvos, being organic in nature, may interfere or disrupt the organic membranes of the cells thus causing cell lysis, or they may at specific concentrations enter the cells and affect various organelles of the cells. Loss of certain cells from circulation may be due to their selective adherence to strategic areas (Bang, 1983; Roesijadi and Robinson, 1994). The decrease of small granule haemocytes and large granule haemocytes in the higher concentration of the pesticide may be due to their destruction by lysis. Literature on the effect of pesticides on the immune system, particularly on the cellular factors of crustaceans is limited.

5.2.2.1.2. Humoral factors

The treatment groups showed a decrease in total serum protein concentration compared to control, but among the different test groups, there was an increase in protein concentration. SDS-PAGE of the serum of *F. indicus* revealed greater expression of polypeptide bands in test groups. The expression of polypeptides above 98 kDa molecular weight, was more in the 0.02 ppm. The haemocyanin fraction and the lower weight bands in the weight range of 20 to 56 KDa were more pronounced in the 0.05 ppm exposed animals, than that of the other groups. The haemocyanin band was also expressed in the 0.08 ppm animals. Haemagglutination titers were greater in the higher concentration groups. Of the enzyme profile, the phenoloxidase enzyme increased in the test animals at low concentration of nuvan. But the enzyme activity decreased with increasing concentration. Of the lyzosomal enzymes, the acid phosphatase decreased with increasing concentrations of nuvan, where as alkaline phosphatase increased with pesticide concentration.

The reduced level in the protein concentration at lower levels of nuvan can be due to the disruption of protein synthesis in nuvan exposed animals (Reddy and Rao, 1990; Geraldine *et al.*, 1999). A comparative rise in the protein

level in animals exposed to higher concentrations may be due to tissue breakdown, which was evident histologicaly too. This may release many stress enzymes and other molecules of protein nature. An indication of this was seen on SDS-PAGE. In the test groups there was greater expression of many polypeptide bands, compared to control animals. At low concentration of pesticide, the polypeptide bands of greater molecular weight were intense than that of the control animals. In the higher concentration groups of 0.05 ppm and 0.08 ppm, expression of haemocyanin band and the lower molecular weight bands were intense. This expression of lower weight bands could also be due to the increase in lectins, as seen from the increased haemagglutinin titers in the higher concentration groups (Ratanapo and Chulavatnatol, 1990; Muramoto *et al.*, 1995).

In many crustaceans, differences in the activity of enzymes have been seen under pesticide exposure (Smith *et al.*, 1995). The increased activity of phenoloxidase enzyme in the test animals in the lower concentration of nuvan may be due to a reduction of plasma inhibitors regulating the prophenoloxidase system. But there was suppression in the activity with increase in the pesticide concentration. Such a reduction was seen in *P. stylirostris* during stress from hypoxia (Le Moullac *et al.*, 1998). However in *F. indicus*, the enzyme activity remained more than that of the control group. The partial decrease in the enzyme activity may be attributed to compromise of enzyme synthesis as a result of increased load of pesticide acting on the system.

Lyzosomal enzyme levels were also altered. In many crustaceans, acid phophatase decreases on exposure to organic pollutants as seen in the present investigations (Reddy and Rao, 1990; Geraldine *et al.*, 1999; Bhavan and Geraldine, 2004). In contradiction to this, the alkaline phosphatase increased in *F. indicus* on pesticide exposure. A probability is the release of this enzyme due to organelle destruction. Thus there is alteration of the immune capability of the animals in the wake of pesticide exposure.

5.2.2.1.3. Histology

Low concentrations of Fenitrothion, an organophosphate insecticide did not produce any histological change in the gills and exopodite of *P. japonicus*. But higher sub-lethal concentrations showed changes such as necrosis of gill lamellae and accumulation of particles between the gill lamellae (Lignot *et al.*, 1997). In the freshwater shrimp, *M. kitnensis* degeneration of the respiratory epithelium and severe haemocytic congestion in the gill lamellae were observed due to this organophosphate (Pawar and Katdare, 1984). In the present study, in *F. indicus*, low concentration of nuvan (0.02 ppm) produced changes such as oedema in the gills. Haemocyte accumulation and engorgement of the sinuses of the secondary gill filaments were seen. Exudation was seen in the antennal gland. Haemocyte congestion is a measure for the elimination of toxic substances (Lowe, 1985). More apparent changes were seen at 0.05 ppm concentration of nuvan such as necrosis of antennal gland and hepatopancreas. In *Charybdis lucifera*, exposure to crude oil produced zonal tubular damage in the hepatopancreas. Cells were shrunken in size and showed amorphous mass of cells in the lumen. *S. serrata* also showed zonal tubular damage, but except for the necrotic regions, individual cells were normal (Chandy and Kolwalker, 1984). In the current study cardiac tissue was also affected with loss of striation and focal accumulation of haemocytes in many parts at 0.05 ppm concentration. Haemopoietic tissue exhibited degeneration such as fluid accumulation, and vacant areas around the haemopoetic nodules. At the highest concentration of 0.08 ppm nuvan, hepatopancreas showed complete necrosis. Necrotic changes were also evident in the heart muscle. Changes were also observed in the gill filaments of *P. monodon* on exposure to Gusathion A (Baticados *et al.*, 1990). 'Perfektion' produced heavy damage to the hepatopancreas of *P. monodon*, even though it did not manifest in the behavior of the animal (Vogt, 1987). In *F. indicus* also, nuvan produced noticeable changes in the histology of many of the organs like antennal gland, hepatopancreas, heart and haemopoetic tissue, even at very low levels of 0.02 ppm. Thus even sub-lethal concentration of nuvan is capable of producing substantial alterations in the different tissues. The small

granule haemocytes, presumably the main phagocytic cells, and the phagocytic activity were comparatively low at 0.05 ppm. This observation together with the histological changes suggests that chronic exposure to sub-lethal concentrations of nuvan is detrimental to *F. indicus*. It has been reported that low concentrations of crude oil produces greater mortality, than higher concentrations (Chandy and Kolwalker, 1984). This is due to the stability of the oil at lower concentration. Being an organic pesticide, the same maybe true for nuvan also and has better chances of penetrating the lipid layer of body membranes and interfering with the enzyme systems and other proteins at a low concentration(Marwell and Baker, 1967; Nelson-Smith, 1977). Continuous exposure to low concentrations of the organophosphate nuvan may cause damaging results in the fundamental structure of the tissues, affect the haemocytes and thus, the enzyme systems.

5.2.2.2. Zinc

Among the cellular factors of *F. indicus*, the total haemocyte count was severely affected by zinc exposure and showed significant deviation from that of the control. The total cell count doubled in the animals exposed to 0.1 ppm of zinc. Heavy metal exposure has shown to increase total haemocyte counts and is due to either proliferation of cells or due to migration of cells from tissues to circulation (Pipe *et al.*, 1995; Pipe *et al.*, 1999). Haemocytes are known to sequester the heavy metal absorbed during the detoxification process. Hence, there is a demand for large number of haemocytes during heavy metal toxicity, and hence an increase in haemocytes (Fisher *et al.*, 2000). Thus, the increase in number of haemocytes at sublethal concentrations of zinc indicates an immune response to sequester zinc from the body.

Excess amounts of heavy metals alter the physiology of animals by changing their osmoregulatory abilities. This is so, either by inhibiting enzyme activity, by altering ionic transport across ion-transporting epithelia, affecting changes in electrolytic plasma levels, causing proliferation of plasma cells or by causing increased cell turn-over (Roesijadi and Robinson, 1994). In *F. indicus*, phenoloxidase activity alone showed significant change among the humoral

factors. It decreased to half that of the control. Granulocytes are known to be the carriers of enzymes associated with the prophenoloxidase system (Hose *et al.*, 1987; Hose and Martin, 1989; Tsing *et al.*, 1989). Haemocytes are known to sequester metals and thus the metal might have entered the granulocytes by phagocytosis or pinocytosis for degradation (Roesijadi and Robinson, 1994). The prophenoloxidase system in the haemocytes might have been suppressed, even though the heavy metal was present at a sub-lethal level. Earlier studies have also shown decreased phenoloxidase activity in shrimp on exposure to low concentrations of heavy metals (Yeh *et al.*, 2004). High concentrations of heavy metals in the aquatic medium result in its accumulation in body tissues. Aquatic animals have the ability to regulate the concentration of essential trace elements to a certain body concentration (White and Rainbow, 1982). It is seen that crustaceans have evolved mechanisms to regulate concentrations of essential but potentially toxic metals to a constant body level depending on the metabolic demand (Rainbow, 1985). The heavy metals zinc, copper and manganese were regulated to body limits in *C. maneas* and *H. vulgaris* (Martin *et al.*, 1977; Bryan, 1984) and in *P. elegans* (White and Rainbow, 1982). Temporary absorption and storage in the hepatopancreas, loss across body surface, and excretion through faeces are the main ways of disposing excess concentrations of metal.

5.2.2.2.1. Histopathology

In the present study, in *F. indicus* the proximal part of hepatic tubules in certain regions showed necrosis. Changes were seen in the gills as well. There was enlargement of the lacunae of secondary gill filaments. The haemal sinuses associated with the secondary filaments also showed enlargemaent. In the heart muscle, accumulation of haemocytes was noticed. In *F. indicus*, sub-lethal concentrations of 100 ppb and 300 ppb of zinc produced degenerative changes in the hepatopancreas and gills (Viswanathan and Mannisseri, 1995). In *S. serrata* too, exposure to cadmium and mercury affected the hepatopancreas (Krishnaja *et al.*, 1987). An increase was seen in the number of haemocytes in the heart. All

these changes, even though, did not produce any behavioral variations in the animals, imply that sub-lethal concentrations of even 0.1 ppm zinc can affect tissues of *F. indicus*.

5.2.2.3. Salinity

The total haemocyte count of *F. indicus* although showed an increase at 10 ppt salinity, at 3 ppt the same was low. The decrease in the total count with salinity was also reported in *F. paulensis* and *L. vannamei* (Le Moullac and Haffner, 2000; Wang and Chen, 2005). The total haemocyte count is the reflection of the circulating cells available to the animal for various functions. A reduction in their number implies that these cells are not available for these functions. Similar reductions in the total haemocyte count have been recorded in the face of other environmental stress conditions such as hypoxia in *P. stylirostris* (Le Moullac *et al.*, 1998) and due to captivity in *L. setiferus* (Sanchez *et al.*, 2001). Reduction in the haemocyte number may also be due to an increase in haemolymph volume (Smith *et al.*, 1995).

In the present study, there was an increase in the total haemocyte count at a salinity of 10 ppt. This could be an indication of the animal, being euryhaline, trying to adjust to the salinity variation by producing more haemocytes. But very low salinitites may have resulted in the compromise of the capacity to withstand the stress and thus hindered the ability to maintain the haemocyte production.

Other cellular factors, that showed significant decrease with decreasing salinity was the percentage of small granule haemocytes and the mean percentage phagocytosis. Small granule haemocytes have been associated with phagocyic activity (Hose and Martin, 1989; Tsing *et* al., 1989). The present observation indicates the greater role of small granule haemocytes in phagocytosis and the adverse effect of low saliniy on the immune system.

Percentage of large granule haemocytes showed an increase with decreasing salinity. This increase may have resulted from the conversion of small granule haemocytes in to large granule haemocytes. Large granule haemocytes are the main carriers of the phenoloxidase enzyme, which is an opsonin

(Perazzolo and Barracco, 1993). Rise in percentage large granule haemocytes is perhaps relative, since the total haemocyte count is reduced at this salinity. This may be because haematopoiesis is affected, which resulted in reduced percentage of hyalinocytes and small granule haemocytes

In *F. indicus*, low saline medium results in the reduction of cellular factors like total haemocyte count, small granule haemocytes and the pahgocytic activity. This compromise in the cellular defense factors makes the animals more susceptible to opportunistic pathogens present in the surrounding medium. The chances of susceptibility is more as the solubility of heavy metals in the medium increases with dilution and this adds the chances of susceptibility of the animals.

The total serum protein concentration decreased with decreasing salinity. This was in contradiction to many studies, which showed an increase in the protein concentration with dilution of medium (Gilles, 1977; Vargas-Albores *et al.*, 1998). But in *F. paulensis* and *P. monodon*, the protein concentration decreased in those animals reared in lower salinity waters (Chen *et al.*, 1994; Le Moullac and Haffner, 2000). This may be accounted by the stress that the animals face to maintain equilibrium with the surrounding medium.

SDS-PAGE revealed polypeptide bands of molecular weight more than 98 kDa in animals reared in 10 and 3 ppt waters. The bands were more apparent in the animals at 10 ppt salinity. The haemagglutination titers of animals in 10 ppt waters were also greater than the animals in other salinities. The increase of total haemocyte count in the 10 ppt group may have resulted in increased production of agglutinins. The occurrence of pathogens is usually more in low saline medium. The increase in the agglutinin titers in animals maintained at low saline medium indicates their compulsion to meet the impending attack of pathogens. But the ability to defend may decrease due to stress at very low salinities viz., 3 ppt.

Lowering of phenoloxidase activity of the animals was seen with decreasing salinity. However, acid phosphatase activity was greater in the animals maintained at lower salinities. Although alkaline phosphatase activity decreased at 10 ppt, it showed an increase in the 3 ppt group. Reduction in the

phenoloxidase with decrease in salinity has been reported in *L. vannamei* and *F. paulensis* (Perazzolo *et al.*, 2002; Wang and Chen, 2005). Even though the large granule haemocytes increased in percentage in animals of 10 and 3 ppt salinity, the total reduction in the haemocytes might account for this (Perazzolo *et al.*, 2002). The release of enzymes may be inhibited.

At times of low salinity, the animals require energy to maintain the osmolality of the body fluid. Phosphatases are involved in energy release and the increase in the acid and alkaline phosphatases at low salinities may indicate their task at increasing the energy production in the stressed animals (Lignot *et al.*, 1997).

5.2.2.4. Immunostimulant

5.2.2.4.1. Changes in the cellular factors

The THC of the test animals showed the same trend as that of the control but, displayed lower values. Decrease in the total haemocyte count has been recorded in immunostimulant application (Smith and Söderhäll, 1983; Smith *et al.*, 1984). The decrease in the total count may be due to the adherence of the haemocytes to crucial sites such as gills, which is considered one of the main entry points for pathogens (Smith *et al.*, 1984). The decrease was seen till the third week. The activity of the stimulant may be time bound, and may last for only a certain period and thus may have caused a time bound decrease in the number of circulating cells.

A significant increase in the percentage hyalinocytes and percentage small granule haemocytes was seen at fourth week. Similarly, a rise was observed in the percentage of large granule haemocytes at the end of the experimental period. This indicates that the effect of the stimulant on the various cell types is time dependent.

Although in the initial stages, the phagocytic activity of the animals was lower than the control, there was a significant increase at third and fourth weeks. Phagocytic activity is seen to increase in the application of immunostimulants (Itami *et al.*, 1998 Du *et al.*, 1997). In the present study, the effect of the

immunostimulant could only be seen after a certain period. In *F. indicus* it may take time for the immunostimulant to activate and maintain the phagocytic activity in the animals (Renpipat *et al.*, 2000).

5.2.2.4.2. Changes in the humoral factors

Among the humoral factors, significant increase in haemagglutination, acid phosphatase and alkaline phosphatase was observed after the third week of stimulant incorporation in feed. Increase in the haemagglutinin and acid phosphatase activity of the serum has been reported in *M. rosenbergii* (Sung *et al.*, 2000; Mo *et al.*, 2000; Kumari *et al.*, 2004). In *F. indicus* also, inclusion of immunostimulant in the shrimp feed indicated a stimulation of humoral factors, particularly the agglutinins and phosphatases on long term application, implying that the activity of immunostimulants on the defense factors of *F. indicus* is time dependent.

5.3. Conclusion

The present investigation revealed three types of circulating haemocytes in the haemolymph of *F. indicus*: hyalinocytes, small-granule haemocytes, and large-granule haemocytes. Intermediate stages indicate the maturing process of a single cell. The presence of enzymes such as peroxidase, phenoloxidase and acid phosphatase in the haemocytes, and the substantial production of oxygen radicals during phagocytosis show that the haemocytes are capable of mounting a fine cellular defense mechanism. The enzyme activities of the serum and the presence of agglutinins in the serum, which may act as opsonins, agglutinate foreign particles and augment phagocytosis, confirm the presence of a superior humoral immune system in *F. indicus*

Bacterial infection caused considerable variations in the cellular and humoral factors, such as the number of circulating cells and haemagglutinating activity, especially in the initial hours of infection. The total haemocyte count, haemagglutination titer and phenoloxidase enzyme showed significant reductions on bacterial presence and could be used as indicators of bacterial infection.

The number of circulating cells showed drastic fluctuation on exposure to pollutants. Nuvan at low concentrations was able to produce changes in the haemolymph factors and in the tissue organization, which implies that the animal is under stress and is easily prone to infections. Exposure to nuvan resulted in significant variation in all of the cellular and humoral factors, especially, the total haemocyte count, percentage of small granule haemocytes, phagocytic activity and the haemagglutinating activity, which might be good indicators of pesticide pollution. Heavy metal exposure caused significant increase in total haemocyte count and reduction in phenoloxidase enzyme activity Even changes in the physio-chemical parameters, such as salinity caused fluctuations in the defense factors, indicating stress in this euryhaline species. The dietary incorporation of a commercial immunostimulant containing β-1,3 glucan resulted in stimulation of some of the humoral defense factors of *F. indicus*, but was time dependent. The modulations, on exposure to various external factors, in the cellular and humoral factors, especially, total haemocyte count, phagocytic activity, haemagglutinating activity and the phenoloxidase and acid phosphatase enzymes suggest that these parameters could be used as indicators of the health status of *F. indicus*, which assist in better monitoring and effective health management of this important cultured species.

Summary

6. Summary

1. The haemocytes of *F. indicus* comprise of hyalinocytes, small granule haemocytes and large granule haemocytes. The main distinguishing feature between the various haemocytes is the presence or absence of small or large, electron dense granules. The presence of intermediate stages indicates a maturing series of cells.
2. Haemocytes of *F. indicus* possesses carbohydrates and lipids, and the enzymes prophenoloxidase, peroxidase and acid phosphatase.
3. Mean total haemocyte count of apparently normal *F. indicus* is 1978.77 ± 4.28 $\times 10^4$ cells/ml of haemolymph. The small granule haemocytes are the predominant type, followed by hyalinocytes and large granule haemocytes.
4. The cells of *F. indicus* are highly phagocytic showing a mean phagocytic activity of 53.15 ±8.87%, and involve the production of reactive oxygen intermediates. Thus, in *F. indicus*, oxygen radicals play an important role in foreign body elimination.
5. Among the humoral factors, the mean total serum protein is 55.55 ± 12.57 mg/ml. The mean phenoloxidase activity is 1.164 ± 0.59 enzyme units, the mean acid phosphatase activity is 33.59 ± 7.5 KA units, and the mean alkaline phosphatase activity is 2.309 ± 0.318 KA units. The haemagglutinating activity (reciprocal of titer) is a mean of 33.77 ± 18.66.

6. In *F. indicus*, the bacterial injection caused reduction in the total count of the haemocytes. These changes were time dependent. The individual types of haemocytes also showed time bound changes.
7. Increase in the haemagglutinating activity and the phenoloxidase enzyme in the serum of the animals was observed on exposure to bacteria. The serum protein concentration and the activity of acid phosphatase enzyme also showed an initial increase indicating an immune response.
8. Low concentrations of nuvan resulted in reduction of the total haemocyte count of the animals. But the haemocyte number exhibited remarkable increase as the concentration of the pesticide increased.
9. Sub-lethal concentration of pesticide altered the percentage of small granule haemocytes. At 0.05 ppm of nuvan, a reduction in the number of small granule haemocytes and a corresponding fall in the phagocytic activity were observed. This indicates the role of these haemocytes in phagocytosis and the effect that pollutants have at sub-lethal levels.
10. There was a reduction in the total serum protein of the animals on exposure to nuvan. But increasing concentrations of the pesticide resulted in a slight increase in the protein concentration, which may be caused by tissue damage.
11. Although the phenoloxidase activity increased on exposure, it decreased with increasing concentration of pesticide. The increase in the enzyme activity may be due to a reduction of plasma inhibitors regulating the

prophenoloxidase system. The alkaline phosphatase concentration increased with nuvan exposure.

12. Sub-lethal concentrations of nuvan resulted in necrotic changes in the various tissues of the animals.
13. The heavy metal zinc, at sub-lethal levels caused an increase in the circulating cells, indicating a demand for them in order to sequester the metal.
14. Zinc, at sub-lethal level, suppressed the phenoloxidase activity of the animals.
15. Histology of the tissues revealed significant changes in their organization on exposure to sub-lethal concentration of zinc.
16. Decrease in salinity resulted in the increase in total haemocyte count, indicating the attempt of the animals to meet the stress of salinity reduction. But extreme reduction in salinity resulted in a decrease in the total number of circulating haemocytes.
17. Salinity reduction of the medium affected the granulocytes of the animals. The small granule haemocytes decreased with decreasing salinity, where as the large granule haemocytes increased with salinity reduction.
18. Reduction in salinity had an impact on the phagocytic activity of the animals. It decreased with decreasing salinity.
19. Total serum protein showed a significant reduction with decreasing salinity.

20. The haemagglutinating activity was greater in animals reared in low salinity medium as a probable way of increasing the defenses of the animals.
21. The acid phosphatase activity of the animals increased with decrease in salinity. Alkaline phosphatase activity displayed increase at very low salinity. Phenoloxidase enzyme also decreased with decreasing salinity, but was not significant.
22. The commercial immunostimulant 'Allways' stimulated the humoral factors of *F. indicus*, especially the haemagglutinating activity, but the effect was time dependent.

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