Discussion

The epizootic ulcerative syndrome of fish is at present the most common and dreadful disease of various types of fresh and brackish water fish in India. A large amount of research work is being conducted to identify the exact etiology of the disease.

In North Bengal, the disease has been occurring every year during a definite period of the year, i.e. generally from October to March. In the present study an attempt has been made to know whether some physico-chemical parameters of pond water have any correlation with the EUS outbreak and to find out the effect of pathogenic bacteria and fungus isolated from the ulcers of EUS affected fish of North Bengal.

The results of the studies on water parameters clearly showed (Sec-4.1) that different physico-chemical factors of the pond water in three different places of the disease prone areas of North Bengal were variable during the three years study period. Generally, the outbreak of the disease starts in the month of October and persists upto March. In one occasion the diseased fish were collected in the last week of September 1998 and in other case in the last week of April 2001. The results showed that during the outbreak of the disease, there were significant declining trends of three factors e.g. hardness, total alkalinity and pH values of the pond water. Variations of surface temperatures of the pond water in three areas were also notable. Fall of surface temperature was noticed in all three ponds in three different areas during the onset of the disease. Water temperature remained below 30°C most of the time of the year. Only for a short period of time it goes above 30°C. This temperature condition is favourable for the growth of *Aphanomyces* sp. associated with EUS. The pH of the pond water remained slightly acidic during most of the time of the year (September - October to March). The dissolved oxygen content of pond water remained low in all the three ponds from July' 96 to Sept. 96. Most probably this was due to flood which broke out in the month of June, 1996. The geographical position of North Bengal is unique. Most of the large rivers and its tributaries originate from the Himalayas. Some areas of North Bengal witness flood almost

every year during rainy season. The dissolved oxygen content of one pond (Salbari) in the month of July 1996 was very low only (2.6 ± 0.163). The depletion of oxygen might be due to the entry of flood water.

In Assam EUS outbreak was first reported after flood in the month of May 1988 (Das, 1988). In Sunderbans after a devastating cyclone in 1988 EUS was detected (Saha *et al.,* 1992). In cauvery river system in Karnataka EUS first occured in 1991 immediately after flood (Mohan and Shankar, 1994). Abdul Hameed (1996) reported the EUS outbreak for four consecutive years (1991-1994) in Karnataka after major flood.

Flood in Bangladesh in 1988 resulted in the rapid spread of EUS (Barua, 1992).

Rodgers and Burke (1981) expressed that EUS prevalence in estuarine fish was due to stress by low or rapidly changing water temperature. Roberts *et al.* (1986) expressed that EUS out break occured in cyclic manner when the temperature fall after heavy rainfall. The incidence of disease outbreak was high in water of low alkalinity and hardness (Jhingran, 1990; Das, 1997). Lilley *et al.* (1992) observed that the EUS outbreak was correlated with the lowering of temperature, together with periods of heavy rainfall, low alkalinity and pH fluctuations. Zachariah (1992) mentioned that the significant changes noted in the water quality parameters of the EUS zone may cause stress leading to the outbreak of EUS. Bondad-Reantaso *et al.* (1992) observed in Philippines that there were variations in temperature, chloride, rainfall and hardness of water at the time of EUS outbreak. Das and Das (1993) reported that in West Bengal, the disease outbreak occurs after the monsoon when the water temperature falls. Mohan and Shankar (1994) mentioned that barring few exceptions, the disease appeared to occur during colder months of the year or with decreasing water temperature. Chinabut *et al.* (1995) from their experimental studies by challenging snakehead, *C. striata,* with spores of *Aphanomyces* sp. associated with EUS showed that mortalities were high when water temperature were low.

Callinan *et al.* (1995a) reported that in both Philippines and Australia, EUS outbreak took place in estuarine fish only from estuaries having significant areas of sulphate soil in their catchments. Localised EUS outbreaks were reported after a rainfall and was associated with pH values between 5 and 6.3. Lumanlan-Mayo *et al.* (1997) suggested from the field and laboratory experiments that low water temperature <30°C played a significant role in EUS outbreak. They also showed that rainfall and decrease in alkalinity and calcium and magnesium hardness were associated with the disease outbreak in *C. striata*, but at the onset of winter, as the temperature began to decrease, artificial maintenance of high levels of alkalinity and hardness failed to prevent the disease outbreak. Chinabut *et al.* (1995) suggested that the seasonality of EUS was due to immunosuppression at low temperature. Ahmed and Hoque (1998) observed that water quality parameters such as temperature, alkalinity and hardness were reduced in December, January and February in comparison to other months. Sanaullah *et al.* (2001) concluded that rapidly declining seasonal temperature and changing water quality, particularly lower chloride and alkalinity with respect to hardness might cause severe stress on immune response and reduce the resistance to pathogen which ultimately may result to EUS. Pathiratne and Jayasingha (2001) suggested that declining dissolved oxygen concentration in water coincided with initiation of EUS outbreak in Bellancoila. Attidiya Wetlands in Sri Lanka.

So from the above discussion and from the results of the experiment conducted for three years from July 1996 to June 1999 on the water parameters of the pond water of the diseased prone areas it can be concluded that declining temperature and pH of water may be associated with the repeated outbreaks of EUS in some areas of North Bengal.

During the entire study period 234 EUS affected fish of different species were collected (Sec-4.2). The ulcers were categorised into three major types i.e. superficial, moderate and severe depending on the nature of ulcer (Sec-4.3). Out of 234 infected fish collected 40 were with initial stage of ulcer, 44 with moderate ulcer and 150 with advanced stage of ulcer. The

histopathological observations in ulcer of different fish showed loss of epidermis, granulomatous changes and necrosis of the muscle. In the present experiment, five species of naturally infected fish were observed histopathologically. The ulcer of all that fish showed the presence of non-septate invasive fungal hyphae (Sec. 4.4) which were stained black with Grocott's methenamine silver stain (GMS) and pink with PAS stain. In some cases, the hyphae were surrounded by macrophage-like cells. The sections of severe ulcers showed the complete loss of epidermis and granuloma formation with necrotic changes in the dermis and the underlying musculature. In some ulcer sections, budding of fungal hyphae was also found (Fig.32). Sections of the liver of infected fish showed the presence of vacuolation of the hepatocytes and infiltration of blood capillaries. Sections of the kidney showed tubular vacuolation and necrosis in some regions (Sec-4.4).

Callinan *et al.* (1989) described EUS of mullets in Australia and grouped these into four types: erythromatous dermatitis, intermediate type, necrotizing dermatitis and dermal ulcer. Viswanath *et al.* (1997) categorised EUS lesions into three types e.g. Type-I (early lesion), Type-II (moderately advanced lesion) and Type III (advanced lesion). Chinabut and Roberts (1999) Classified EUS into five types depending on clinical patterns : Type I, Type-II, Type-III, Type-IV and Type-V.

Kumar *et al.* (1991) reported the complete loss of epidermis of the skin at the ulcer area where dermis and hypodermis showed characteristic cyst-like or nodule-like granulomatous formation. The muscle layers also showed granuloma formation. The sinusoidal spaces and blood vessels were congested in the liver tissues and plenty of lymphocytes were present in the liver parenchyma. The haematopoietic tissue showed proliferation of macrophage cells which indicated the inflammatory response. Qureshi *et al.* (1995) found that the epidermis of the infected *C. batrachus* were destroyed completely and wherever it existed showed spongiosis. The dermis lost its original compactness.

Mohan and Shankar (1995) reported the presence of fungal hyphae with severe necrosis of the dermis and epidermis. They also reported that fungal hyphae were surrounded by one to several layers of macrophages and invested by fibroblasts. Cruz-Lacierda and Shariff (1996) reported the association of highly invasive broad (upto 24.6 μm in diameter) branched aseptate fungal hyphae.

Viswanath *et al.* (1998) suggested that fungal hyphae invade the muscle in all directions from the centre of the dermal tissue. The ulcer in the advanced stage showed mycotic granulomatous responses and massive necrotic changes associated with fungal invasion in the integument and skeletal musculature. They also reported invading fungal hyphae in the abdominal viscera and fungal granuloma in the kidney, liver and digestive tract. But in this experiment no fungal invasion in liver, kidney and spleen was found.

In the present investigation naturally infected fish showed low TEC and Hb content in comparison to that of healthy fish (Table 12). The shape of the RBC changed to round and vacuolatlion in the nucleus and cytoplasm of the RBC were also observed. Disintegration of nucleus were prominent. Presence of bacteria was detected in the smear preparation of blood of infected fish. In the experimentally infected fish significant decrease ($P<0.01$) in TEC content were observed after 72 h of P_3 and C_2 and 96 h of P_1 and M_2 bacteria inoculation respectively.Hb content also decreased significantly ($P<0.01$) in experimentally infected fish (Table 24).

Pradhand and Pal (1992) observed significant decrease in haemoglobin content and erythrocyte count in *C. punctatus* after 24 and 48 h of inoculation of three pathogenic bacteria R_1, R_2 and R_3. Saha (1998) also observed similar type of results of TEC and Hb. content in experimentally injected *H. fossilis.* Das and Das (1993) observed that there was a decline in counts of RBC followed by a drop in haemoglobin content and hematocrit values which indicated anaemic condition in the naturally infected *C. mrigala* Prasad and Qureshi (1995) reported remarkable changes in the number of RBC and Hb content in

fish affected with EUS. Pathiratne and Rajapakshe (1995) observed low TEC and Hb content in EUS positive fish and suggested that the anaemic condition resulted from loss of blood and destruction of erythrocytes.

Decrease in the number of erythrocyte count and Hb content were reported in case of other diseases of fish caused by *Pseudomonas* sp. and *Aeromonas* sp. Takahashi and Kusuda (1979) observed that the erythrocyte count in induced scale potrusian disease by *A. liquefaciens* in coloured carp was decreased with lapse of time. They concluded that the cause of decrease in the number of erythrocytes was due to enormous disintegration of erythrocytes by the infection. Barham *et al.,* (1980) found that bacterial infection caused significant reduction in total erythrocyte count and haemoglobin concentration in rainbow trout, *Salmo gairdneri* Richardson.

Takahashi (1984, a, b) reported marked decrease in TEC and Hb levels in experimentally infected corp treated with *A. hydrophila* and suggested that the erythrocytes were destroyed by haemolysin produced by *Aeromonas* sp. entering the blood and there was a decrease in the haematopoietic function of kidney and spleen due to necrosis in these organs.

In this study there were large number of disintegrated erythrocytes and lowering of TEC and Hb content among naturally infected and experimentally infected fish. This indicates that bacteria from skin ulcers entered into blood and caused the destruction of erythrocytes. Such was reflected in the lowered TEC and Hb content values.

The morphological features and biochemical profiles of the isolated pathogenic bacteria, P_1, revealed (Sec. 4.6) that these bacteria were gram negative motile, catalase positive, oxidase positive, utilised glucose oxidatively and produced yellowish green pigment in King's B medium (Table 13 & 14). Thus, it belonged to the genus *Pseudomonas* (Stanier *et. al.* 1966, Palleroni 1984). Furthermore, Pigment production, inability to grow at 42°C and acid production from sucrose indicate that P_1 resembled *P. fluorescens.*

P_3, C_2 and M_2 isolates were gram negative straight rods, motile, catalase positive, oxidase positive, indole positive and utilised glucose by fermentation (Table 13, 14, 15, 16, 17 & 18). These belonged to the genus *Aeromonas* (Popoff 1984). However, these were not considered to belong to the genus *A. salmonicida* because these can grow at 37°C while *A. salmonicida* could not. The P_3 isolate phenotypically resembled *A. hydrophila* as it produced gas from arabinose. The C_2 isolate resembled *A. sobria* as it differed in M-R and V-P test with that of P_3 and did not produce gas from arabinose. The isolate, M_2 did not produce gas from glucose and produced acid from L-arabinose, thus, resembling *A. caviae.* The biochemical profile of P_2, M_1 and C_1 showed that these bacterial isolates belonged to the genus *Micrococcus.* These differed from the genus *Staphylococcus* and *Streptococcus* with respect to the breakdown of glucose. As, these isolate utilized glucose oxidatively and produced a yellowish pigment, P_2, M_1 and C_1 resembled Micrococcus. The sphere-shaped bacterium, C, was gram positive, non-motile, non spore-forming, catalase positive, indole negative, oxidase negative and oxidative, occurring singly, in pairs, in tetrad, in short chain or in irregular cluster (dividing in more than one plane). Colonies were yellow and small; smooth, convex. It satisfied the characteristics of the species *Micrococcus varians* (Kocur, 1986) e.g. oxidase negative, oxidative in metabolism, reduction of nitrate and nitrite, good growth between 25-37°C, and non pathogenic. It differed from *Staphylococcus aureus* with respect to carbohydrate metabolism. *S. aureus* was fermentative and the *M. varians* was oxidative. Hence, it belonged to *M. varians.* From the morphological and biochemical profile P_4 isolate resembled *Moraxella.* (Bøvre, 1984a) being oxidase and catalase positive which distinguished it from *Acinetobacter* sp. and *Kingella* sp. which are two other genera of the family Neisseriaceae (Bøvre, 1984b). The genus *Neisseria* is also catalase and oxidase positive but morphologically they are coccoid (Vedros 1984).

- Altogether, one *Pseudomonas* sp., three *Aeromonas,* three *Micrococcus* sp. and one *Moraxella* sp. were isolated from the infected *C. mrigala, Puntius* sp. and *C. gachua.* Out of these eight bacteria one *Pseudomonas* sp. and

three *Aeromonas* spp. were pathogenic and produced ulcer when injected intramuscularly.

Globally, *Aeromonas* sp. is one of the most common bacteria associated with fish diseases. Although many strains are regarded as opportunistic pathogens, others are clearly primary pathogens in their own right (Trust, 1986). Fish diseases which involve *A. hydrophila* include motile Aeromonad septicemia (AFS, 1975), red spot disease of European eel, *A. anguilla* (Schäperclaus, 1934), red disease of Japanese eel, *A. japonica* (Hoshina, 1962) and red disease of carp, *C. carpio* (Egusa, 1978). Jo and Onishi (1980) isolated *A. hydrophila* from all diseased, cultured ayu, *P. altivelis,* characterized by exopthalamus and subcutaneous ulceration. Rahim *et al* (1985) isolated *A. hydrophila* from the wounds of five species of fishes in Bangladesh. Okpokwasili and Okpokwasili (1994) found that *Pseudomonas* spp. and *A. hydrophila* isolated from brown patch disease of tilapia were more virulent to tilapia fingerlings when infected by a mixed culture than *A. hydrophila* or *Pseudomonas* spp. alone. Esteve *et al.* (1993) isolated *A. hydrophila* and *A. jandaei* from diseased European eel (*A. anguilla*) from an eel farm in Spain which caused ulcer disease by intraperitoneal injection (LD_{50} dose : $10^{5.4}$ to $10^{7.5}$ cfu/ fish) and also by bath exposure to 10^{7} to 10^{8} cfu/mL in healthy eels.

The goldfish ulcer disease (GUD) is caused by an atypical strain of *A. salmonicida* which is responsible for loss of both wild and cultured goldfish (Trust *et al.,* 1980; Whittington *et al.,* 1987).

Aeromonas salmonicida has been reported to be isolated form head ulcer of eel, *Anguilla japonica* characterized by ulcerative lesion on the head (Ohtsuka *et al.,*1884) and carp erythrodermatitis characterized by cutaneous ulcerative lesion (Csaba *et al.,* 1984).

Aeromonas liquifaciens, another pathogenic strain of *Aeromonas* sp. was isolated from scale protrusion disease in carp by Kusuda and Takahashi (1970), which affected fish farms of Japan. Rainfection studies in carp and gold fish showed that percent mortality of carp was higher than that of gold fish.

A. punctata has been regarded as the etiological agent of infectious dropsy in carps by Schäperclaus (1965) and the thought that primary infections resulting in disease may be induced by *P. fluorescens*. Later he indicated that 3 different microorganisms, *A. punctata, P. fluorescens* and a virus may be involved in this disease which manifest itself in several forms (Schäperclaus, 1969).

Different species of *Pseudomonas* has been reported to be the causative agent of various fish diseases throughout the world. *P. anguilliseptica* was identified as the etiological agent of red spot disease in japan characterized by petecchial haemorrhage in the mouth, opercula and ventral portion of the body of the fish (Wakabayashi and Egusa, 1972; Muroga *et al.,*1973; Jo *et al.,* 1975; Nakai *et al.,* 1985). *P. anguilliseptica* was also isolated from red spot disease of pond cultured eel, *A. japonica* in Taiwan (Kuo and Kou, 1978), from *A. anguilla* in Scotland (Nakai and Muroga, 1982; Stewart *et al.* 1983) and from salmonid fish in Finland (Wiklund and Bylund, 1990). Muroga and Nakajima (1981) reported artificial induction of red spot disease in *A. japonica* with *P. anguilliseptica* and was able to induce the same clinical signs as those observed during disease outbreak by reinfection tests.

Pseudomonas fluorescens has been reported to cause haemorrhagic septicemia in European eel, *A. vulgaris* (Andre *et al.,* 1970), pond cultured tilapia, *Sarotherodon niloticus* (Miyashita, 1984; Miyazaki *et al.,* 1984), yellowtail *Seriola quinqueradiata* (Kusuda, 1980) and cyprinid fishes (Bullock and Mc Laughlin, 1970; Shiose *et al.,*1972). Sakai *et al.,*(1989) isolated *P. fluorescens* from diseased rainbow trout *Onchorhinchus mykiss* in Japan and found the bacteria to be pathogenic to rainbow trout and to tilapia (*S. niloticus*). Saeed *et al.*(1987) isolated *P. putrefaciens* from diseased rabbit fish, *Siganus rivulatus* in Red sea. Pal and Pal (1986b) reported induction of ulcer in *A. testudineus* by mixed culture of two bacteria, one fluorescent Pseudomonad and another coccus, *M. varians.*

Pseudomonas fluorescens has been reported to cause haemorrhagic septicemia in European eel, *A.* vulgarise *(Andre* et. al. 1970) and in pond

cultured tilapia, *S. niloticus* (Miyashita, 1984, Miyazaki *et. al.* 1984). Different species of *Pseudomonas* had been reported to be the causative agent of various fish diseases, throughout the world. *P. anguiliseptica* which caused haemorrhage in the mouth, opercula and ventral portion of the body of the fish was identified as the etiological agent of red spot disease in Japan (Wakabayashi and Egusa, (1972); Muroga *et. al.* (1973); Jo *et al.* (1975), Nakai *et al.* (1985), Rahim *et. al.* (1985) observed that *A. hydrophila* was associated with the wounds of five species of fish in Bangladesh.

Llobrera and Gacutan (1987) reported the isolation of *A. hydrophila* from EUS affected fish. Boonyaratpalin (1989) found primarily *A. hydrophila* and occasionally *Pseudomonas* sp. associated with the outbreak of EUS in Burma, Indonesia, Lao peoples Democratic Republic, Malayasia, Singapore and Thailand. Association of *A. hydrophila* with EUS affected fish in Sri Lanka was also reported (Costa and Wijeyaratne, 1989; Subasinghe *et. al.* 1990). Karunasagar *et. al.* (1989) and McGaray *et. al.* (1991) recovered *A. hydrophila* and *A. sobria* more often.

Two virulent strains of *Pseudomonas* sp. and one virulent Aeromonad, *A. caviae* were isolated from ulcerative air breathing fish from North Bengal in 1988 and reported to be pathogenic to *A. testudineus* (Pal and Pradhan, 1990) and *C. punctatus* (Pradhan and Pal, 1990).

Besides *Aeromonas* sp. and *Pseudomonas* sp. some other types of bacteria were also found to be associated with EUS, Micrococcus sp. (Jhingran and Das 1990), *E. Coli* and *P. aerouginosa* (Kar *et. al.* 1990) CAN bacteria (Chakraborty and Dastidar, 1991). Chattopadhyay *et al.* (1990), McGaray *et al.* (1991), Lio-Po *et al.* (1992, 1998), Torres *et al.* (1993), Cartwright *et al.* (1994) also reported the association of mainly *Aeromonas* sp. and *Pseudomonas* sp. with EUS. Ali and Tamuli (1991) isolated three types of bacteria from ulcers of four species of affected fish and reinfection studies showed that *Aeromonas* sp. produced only mild infection. *Vibrio* sp. induced similar types of disease signs while *Micrococcus* sp. failed to induce any sign.

Mukherjee *et al.*, (1991) isolated five distinct strains of *A. hydrophila* from EUS affected fish. Torres *et. al.* (1993) isolated 54 strains of *Aeromonas* sp. from EUS affected fish. Karunasagar, *et al.* (1995) isolated *A. sobria* and *A. hydrophila* from EUS affect fish of Karnataka, India. Aeromonads and Pseudomonads, isolated from EUS affected fish, were found to induce EUS like lesion when injected intramuscularly to healthy snakehead *(O. striatus)* and walking catfish (*C. batrachus*) (Lio-Po et. al. 1992; Leaño *et al.* 1995). Prasad *et. al.* (1995) observed that *C. mrigala* injected with virulent *A. hydrophila* strain isolated from EUS affected *M. armatus* exhibited signs of EUS. Qureshi *et. al.* (1995b) isolated nine types of bacteria from EUS affected fish out of which *Pseudomonas* sp. and *Aeromonas* sp. were found to be highly pathogenic while Micrococcans and Cytophagans were less pathogenic. Lio-Po *et. al.* (1998) isolated four types of bacteria associated with EUS, namely *A. hydrophila, Aquaspirillum* sp. *Pseudomonas* sp. and *Streptococcus* sp. Out of these bacteria, *A. hydrophila* was highly pathogenic, inducing dermomuscular lesions in both intramuscularly injected *C. batrachus* and *O. striatus. Streptococcus* induced moderate and slight ulcers in *C. batrachus* and *O. striatus,* respectively. *Pseudomonas* sp. *and Aquaspirillum* sp. were slightly pathogenic to *C. batrachus* only.

Saha and Pal (2000) isolated 16 strains of bacteria from *C. punctatus, Puntius* sp. and *Mystus* sp. belonging to the genus *Pseudomonas, Aeromonas, Micrococcus, Bacillus, Vibrio* and *Moraxella.* Among these bacteria only six strains of aeromonads and pseudomonads were pathogenic and the rest were non-pathogenic. Saha and Pal (2000) showed that four Aeromonads out of sixteen strains induced ulcers in healthy *A. testudineus* when injected intramuscularly.

Nile tilapia (*Oreochromis niloticus*) was found to be resistant to EUS (Ahmed and Rab, 1995). The relative succeptibilities of various other fish species to several bacterial pathogens have been investigated by several workers (Plumb and Sanchez, 1983, Plumb and Hilge, 1987).

Pal and Pradhan (1990) isolated *A. caviae, McGarey et. al.* (1991) isolated *A. hydrophila* and *A. sobria*, Torres *et. al.* (1993) isolated 54 strains of *Aeromonas* sp. Karunasagar *et. al.* (1995) isolated *A. sobria* and *A. hydrophila*, Lio-Po *et. al.* (1992;1998) isolated *A. hydrophila* along with other bacteria. In the present studies *A. hydrophila, A. sobria* and *A. caviae* were isolated from different infected fish which produced ulcer when injected intramuscularly to healthy fish.

From the experimental observations and the above discussion, it can be concluded that out of eight bacterial strains isolated from infected fish three *Aeromonas* spp. and one *Pseudomonas* sp. are pathogenic.

An aseptate fungus (Sec. 4.7) was isolated from EUS affected *C. mrigala* in 1999. The fungus formed sporangia at the hyphal tip not wider than hypha in GPY medium and ball of spores was noticed at the tip of the sporangium. The fungus did not grow at 37°C. From these characteristics, it appeared that the isolated fungus was *Aphanomyces* sp. But unfortunately the fungus could not be maintained *invitro* for more than three months. However, in the month of November 2002, again an aseptate fungus with all the characteristic of *Aphanomyces* sp. was isolated from the ulcer tissue of naturally infected *C. gachua.* To date the fungus is maintained in the laboratory.

Experimentally, the fungal zoospores induced ulcer in healthy *C. punctatus* and produced typical granulomas in the dermis and underlying musculature. Presence of the fungus was also detected in the sections of the ulcer of the experimental fish (Section 4.9 and 4.11).

Roberts *et al.* (1993) reported that a survey of fish affected with epizootic ulcerative syndrome taken from outbreaks in countries throughout South and Southeast Asia showed constantly the presence of a morphologically typical fungus within the lesion. When mycelium from pathogenic isolates of *Aphanomyces* sp. was placed below the dermis of healthy fish, it caused an inflammatory response and proceeded to migrate down into the tissues of fish inducing severe myonecrosis with chronic epithelial reaction. Chinabut *et al.*,

(1995) inoculated snakeheads, *C. striata* (Bloch), with a spore suspension of *Aphanomyces* sp., isolated from EUS affected fish in Southeast Asia. Fish were held at three different temperatures : 19, 26 and 31°C. In the early stages of the disease degenerative changes were observed in all samples, but inflammatory infiltrate was much more marked in fish kept at 26°C and 31°C while fish kept at 19°C developed a severe invasive myonecrosis with limited macrophage response. From 14 to 28 days, post-infection healing become well established at 26° and 31°C and surviving fish kept at these temperatures recovered completely by 28 days. The lesion was still progressing at 21 days post-injection in fish kept at 19°C and all such fish succumbed by this time. The mortalities in the fish kept at 19°C were considerably higher than in the group of fish kept at 26 and 31°C. The findings explain the mortalities from EUS occuring when water temperatures are low.

Lilley and Roberts (1997) reported that a distinct species of *Aphanomyces* was responsible for much of the characteristic pathology of epizootic ulcerative syndrome. Zoospores of 58 fungal isolates were injected intramuscularly in snakehead fish, *C. striata* (Bloch). These fungi comprised *Aphanomyces* strains isolated from EUS affected fish; saprophytic *Aphanomyces, Achlya* and *Saprolegnia* spp. from contaminated waters; and further saprolegniaceous fungi involved in other diseases of aquatic animals. Only the *Aphanomyces* strains isolated from fish affected by EUS. Australian red spot disease (already considered synonymous with EUS) or mycotic granulomatosis described from Japan were able to grow invariably through the fish muscle and produce the distinctive EUS lesions.

Several workers reported the association of *Aphanomyces* sp. with fish disease. Srivastava (1979) reported *Aphanomyces* sp. infection in *C. batrachus,* in Uttar Pradesh, India in 1973, long before the outbreak of EUS in India. Noga (1994) reported that ulcerative mycosis (UM) in USA was caused by *Aphanomyces* sp. Blazer *et al.*, (1999) reported *Aphanomyces* sp. as a cause of ulcerative skin lesion of Atlantic menhaden. Shaheen *et al.,* (1999)

reported that EUS like ulcerated disease was caused by *Aphanomyces* sp. in Egypt. Vogelbein *et al.* (2001) reported from histological observations that ulcers of menhaden harbovred a deeply invasive, highly pathogenic fungus now known to be *A. invadans.*

In Japan, the ulcerative disease of fish was named as mycotic granulomatosis (MG) (Egusa and Masuda, 1971) and *A. piscicida* was isolated from cultured ayu (Hatai *et al.,* 1977; Hatai, 1980). In Australia the ulcerative disease, named as red spot disease (RSD) broke out in the year 1972 (McKenzie and Hall, 1976). Fraser *et al.* (1992) isolated an *Aphanomyces* sp. from the disease. In Asia-Pacific region the ulcerative disease of fish was named as epizootic ulcerative syndrome (FAO, 1986). Pathogenic *Aphanomyces* sp. was isolated by several workers from the EUS affected fish. Willoughby *et al.,* 1994, Paclibare *et al.,* 1994; Callinan *et al.,* 1995a; Lilley and Roberts, 1997; Lumanlan-Mayo *et al.,* 1997). The fungus was named *A. invadaris* (Wiloughby *et al.,* 1994). The protein banding profiles (Callinan *et al.,* 1995b, Lilley *et al.,* 1997b), growth Characteristic (Lilley and Roberts, 1997), Chemical susceptibility (Lilley and Inglis, 1997) and genetic fingerprinting techniques (Lilley *et al.,* 1997) showed that various *Aphanomyces* sp. isolated by various workers were all very same and *A. invadaris* was renamed to *A. invadans* (David and Krik, 1997). EUS is now recognised to be synonymus with MG and RSD (Chinabut and Roberts, 1999).

From the foregoing discussion and from this study, it can be concluded that an aseptate fungus which is capable of growing by budding within the tissue of affected fish exerts the pathological changes in the infected fish.

Chinabut *et al.* (1995) showed that at 19°C the mortality rate of the fish inoculated with zoospores of *Aphanomyces* sp. was 40% and at 26°C the mortality was 12%. But in this work the mortality rate of the fish kept at 25±1°C and inoculated with zoospores of isolated *Aphanomyces* sp. was 30% (Section 4.9).

Saha (1998) showed that the mortality rate of *A. testudineus* inoculated with isolated bacteria R_1, R_2 and R_3 were 39%, 40% and 40% respectively. In the experiment (Section 4.8) it was observed that the mortality rate of *H. fossilis* inoculated with isolated bacteria P_1,P_3 C_2 and M_2 were 20%, 40%, 10% and 23% respectively.

The motality rate of fish in the initial outbreak of the disease was much more than that of the experimentally inoculated fish either by isolated bacteria or by zoospores of isolated fungus.

The histological section of ulcer of bacteria inoculated *H. fossilis* showed some pathological changes in the dermal and muscle layer (Section 4.10) but the section of ulcer of fungal zoospore inoculated *C. punctatus* showed typical granulomatous changes (Sec. 4.11) very similar to the naturally infected fish.

In the naturally infected fish, the TEC and Hb content were low and disintegration of erythrocytes were observed (Sec. 4.5). Similar changes were also recorded in the experimentally infected fish by bacteria (Sec. 4.12).

So from the above discussion and results of the experiments (Sec 4.8 - 4.11) it can be concluded that both bacteria and fungus play important roles in the manifestation of EUS.