CHAPTER VI

DISCUSSION

6. Discussion

The epizootic ulcerative syndrome of fish is at present the most common and dreadful disease of various types of fresh water fishes in Nepal. The disease has been occurring every year during a definite period of year, i.e. generally in winter months in Nepal. 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 eastern Nepal.

The results of the study on water parameters clearly showed that different physico-chemical factors of the water bodies in six different places of the disease prone areas of Eastern Nepal were variable during the two years study period.

Air temperature

The highest air temperature was recorded in the month of March and April in S1 and S2, April and September in S3 and S5, March and August in S4 and May and August in S6. It was mainly due to geographical positions and weather conditions. The minimum air temperature was recorded in the month of December and January in S1, S2, S3 and S4 but in January and February in S5 and S6. When data on monthly air temperature of the whole study period (Nov. 2008- Oct. 2010) were pooled in seasonal values, the highest air temperature was recorded in the summer season followed by rainy season and winter at sites S1, S2, S3, S4, S5 and S6 (Tables 5.19, 5.20, 5.21, 5.22, 5.23 and 5.24). The highest air temperature was recorded 30.56°C at Site 1 in summer and minimum 20.38°C at Site 5 during winter. The summer temperature (30.56°C) was moderate in this region due to its geographic condition. Air temperature showed positive and significant correlation with water temperature at all the sites (Tables 5.3, 5.6, 5.9, 5.12, 5.15 and 5.18). Chakraborty *et al*. (1959), Kant and Anand (1978) and Rawat *et al*. (1995) also obtained strong positive significant correlation between air and water temperatures. The air temperature showed insignificant difference among sites at 5% significance level and significant difference among seasons at 1% significance level (Table 5.26; Figs.5.1,5.10,5.14,5.23,5.27and 5.36).

Water temperature

Generally, water temperature is influenced by air temperature and intensity of solar radiation.

It was highest in summer at all the sites and lowest in winter (Table 5.25). Highest value was recorded in summer might be due to high air temperature and greater light penetration. Though the high air temperature appeared in rainy season, a little lower water temperature was recorded at that time in comparison to that during summer. It might be due to high turbidity, high volume of water and greater velocity of water in rainy season. The water temperature showed positive and significant correlation with free carbon dioxide and biological oxygen demand at all the sites but had inverse and significant correlation with pH, dissolved oxygen, total alkalinity and total hardness at all the sites. Bose and Gorai (1993) reported negative significant correlation between water temperature and dissolved oxygen. Welch (1952) and Munawar (1970) have observed that shallower the water body more quickly it reacts to the change in the temperature. The water temperature was insignificantly different among sites but significantly different among seasons at 1 % significance level (Table 5.27; Figs.5.2, 5.11, 5.15, 5.24, 5.28 and 5.37).

pH

pH of the study sites varied between 6.22±0.309 mg/L during April in the first year at Site 1 and 10.02±0.276 mg/L in February at Site 3 during the study period. The maximum pH of present study was in winter season followed by rainy and summer seasons at all the sites (Table 5.25). The maximum pH in winter season may be attributed to algal blooms because Hutchinson *et al*. (1992) and Roy (1955) have shown that the higher pH is associated with the phytoplankton maxima. The minimum pH recorded in summer may be due to low photosynthesis. Several workers have reported low pH during the low photosynthesis due to the formation of carbonic acid (Hannan and Yong, 1974; Cabecadas and Brogueira, 1987; Bais *et al*., 1995). But Gautam (1990) reported highest pH in summer and lowest in rainy season. The pH showed positive and significant correlation with dissolved oxygen at all the sites. It had positive and significant correlation with total alkalinity at Site 2 but inverse and significant correlation in Site 1. It was inverse and significantly correlated with free carbon dioxide and biological oxygen demand at Site 6, with air temperature and water temperature at Site 3, Site 4 and Site 5 (Tables 5.3, 5.6, 5.9, 5.12, 5.15 and 5.18). Rawat *et al*. (1995) reported positive correlation with total alkalinity (r

= 0.523, P< 0. 05) and inverse correlation with water temperature. The pH showed significant difference among seasons at 1 % significance level (Table 5.28).

Free CO_2

The maximum free carbon dioxide was recorded 179.59 ± 0.332 mg/L in June followed by rainy season and winter season at Site 1, Site 2 and Site 3. Maximum in September and minimum in May at Site 5 and Site 6 but at Site 4, it was maximum in rainy season followed by summer and winter seasons (Table 5.25). The maximum free carbon dioxide was recorded in summer; it may be due to high temperature, high rate of decomposition of organic matter, low volume of water etc. Michael (1969) stated that the concentration of carbon dioxide is directly correlated with the amount and nature of biological activity in water. In this study the minimum free carbon dioxide was found in winter season. Pahwa and Mehrotra (1966), Ray *et al*. (1966), Gautam (1990), and Pandey and Lal (1995) also found minimum free carbon dioxide in winter season.

Free carbon dioxide of water showed positive and significant correlation with water temperature and biological oxygen demand, and inverse and significant correlation with dissolved oxygen at all the sites (Tables 5.3, 5.6, 5.9, 5.12, 5.15 and 5.18). Pahwa and Mehrotra (1966) observed inverse correlation of free CO_2 with dissolved oxygen. The free CO_2 showed significant difference among the sites and the seasons at 1 % significance level (Table 5.29).

Dissolved Oxygen

The dissolved oxygen of the study sites varied between 2.94±0.305 mg/L and 10.16±0.215 mg/L during the study period. It was recorded minimum in the month of August of the first year study period at Site 3 (Table 5.7) and maximum in the month of February of the second year study period at Site 3 (Table 5.8). The maximum dissolved oxygen was recorded in winter season followed by rainy and summer seasons at all the sites. The maximum dissolved oxygen found in winter season may be due to low temperature. Similar observations were made by Moitra and Bhattacharya (1965). The minimum dissolved oxygen was found in summer due to high temperature, and higher microbial demand for oxygen in decomposition of suspended organic matter (Bhowmick and Singh, 1985; Palharya and Malvia, 1988). Elmore and West (1961) stated that an increase in temperature of water resulted in the decrease of dissolved oxygen content of water. Dissolved oxygen content showed positive and significant correlation with total alkalinity

and total hardness at all the sites. It was positively correlated with chloride at Site 1 and Site 4 and positively and significantly correlated with chloride at Site 2 and Site 3. It showed inverse and significant correlation with water temperature, free carbon dioxide and biological oxygen demand at all the sites. Bose and Gorai (1993) also reported inverse and significant correlation of dissolved oxygen with water temperature. Jindal and Kumar (1993) reported inverse correlation of dissolved oxygen with water temperature. According to McColl (1972) the relation between water temperature and dissolved oxygen is not so significant because the production and consumption of oxygen takes place simultaneously. The dissolved oxygen was recorded significantly different among seasons at 1% significance level (Table 5.30).

Biological Oxygen Demand

The BOD of water of the study sites varied between 0.06± 0.062 mg/L and 9.28 ± 0.063 mg/L during the study period. It was maximum in the month of September and minimum in the month of February of the first year study period but maximum in November and minimum in August during the second year study period at Site 1 (Table 5.1). The maximum BOD was recorded in winter followed by summer and rainy seasons at all the sites except Site1 in second year. The maximum BOD obtained in summer may be due to low volume of water and high content of organic matter whereas minimum obtained in winter may be due to low temperature and retarded microbial activity for the decomposition of organic matters. Similar observations were also made by Singh (1995). Ray and David (1966) opined that high BOD value indicates organic waste pollution.

BOD showed positive and significant correlation with air temperature, water temperature and free CO_2 and inverse and significant correlation with pH and dissolved oxygen at all the sites (Table 5.3, 5.6, 5.9, 5.12, 5.15 and 5.18). Ray and David (1966), and Barat and Jha (2002) also reported inverse correlation of BOD with dissolved oxygen. The BOD was recorded significantly different among seasons at 1 % significance level (Table 5.31).

Total alkalinity

The total alkalinity was found maximum in the month of January/February at all the sites. It was found maximum 243.6 ± 0.521 mg/L at Site 1 in the second year study period (Table 5.2) and minimum was 67.68± 0.321 mg/L in Site 2 (Table 5.4). Seasonally the maximum total alkalinity

was found in winter season followed by summer and rainy seasons at all the sites (Table 5.25). It was found maximum in winter season due to high pH. Chakraborty *et al*. (1959), Singh (1990) and Mishra *et al*. (1998) also reported maximum total alkalinity during winter. Water bodies having total alkalinity from 40 to 90 mg/L is considered as medium productive and above 90 mg/L as highly productive (Jhingran, 1991). This investigation showed that the study area is suitable for aquatic production. Total alkalinity showed positive and significant correlation with total hardness and chloride at all the sites. Barat and Jha (2002) also reported positive and significant correlation of total alkalinity with hardness. There was significant ($p<0.01$) differences in values of total alkalinity of water bodies of all sites between months but insignificantly ($p>0.05$) different among corresponding months between first and second years. The total alkalinity was significantly different among sites at 5% significance level and significantly different among seasons at 1 % significance level (Table 5.32; Figs.5.8, 5.12, 5.21, 5.25, 5.34 and 5.38).

Total hardness

The maximum total hardness of water was 196.02 ± 1.976 mg/L at Site 6 (Table 5.17) in the month of June during the second year and minimum 49.5± 0.463 mg/L in the month of December during the first year study period at Site 1 (Table 5.1). The maximum total hardness was in summer season followed by winter and rainy seasons at the Sites 1, 2 and 3 but it was maximum in winter followed by summer and rainy season in Site 4, 5 and 6 (Table 5.25). The maximum total hardness in winter season might be due to low volume of water and slow current of water. Similar results were obtained by Mishra *et al*. (1999). Minimum quantity in rainy season may be due to more dilution of water (Patralekh, 1994). Ruttner (1953) also recorded similar relationship. It showed positive and significant correlation with total alkalinity at all the sites. Total hardness values of water bodies of all sites were significantly ($p<0.01$) different between months but insignificantly ($p>0.05$) different among corresponding months of first and second years. The total hardness was recorded significantly different among seasons and sites at 1% significance level (Table 5.33; Figs.5.9, 5.13, 5.22, 5.26, 5.35 and 5.39).

Chloride

The chloride content of water of the study sites varied between 1.01± 0.093 mg/L and 44.87 ± 0.235 mg/L during the study period. The minimum chloride was recorded in March of the second year study period at Site 4 (Table 5.11) and maximum in April of the first year study period at Site 2 (Table 5.4). Seasonally the maximum chloride content was recorded in summer followed by winter and rainy seasons at Sites 1, 2 and 3 but at Sites 4, 5 and 6 maximum was in rainy season followed by summer and winter seasons (Table 5.25). The maximum quantity of chloride recorded at Sites 2, 3 and 4 in summer season may be due to low volume of water, high temperature and high rate of decomposition of organic matters. Chloride concentration indicates the presence of organic waste of animal origin **(**Thresh *et al*., 1949). Munawar (1970) has suggested that higher concentration of chloride in water is an index of pollution of animal origin and there is a direct relation between chloride concentration and pollution level.

Ganapati (1941, 1943), and Swarup and Singh (1979) also reported an increase in chloride during summer. Minimum quantity of chloride recorded in rainy season might be due to dilution by rain water. But at Site 2 and Site 3 maximum chloride was recorded in winter season which might be due to more contamination by organic matters. Klein (1957) pointed out a direct relationship between amount of chloride and level of pollution. Chloride showed positive and significant correlation with total alkalinity at all the sites. The chloride content was significantly different among sites at 1 % significance level (Table 5.34, Figs.5.7, 5.20 and 5.33).

Turbidity

Turbidity was taken only of rivers which varied between 42.3 ± 0.565 mg/L in first year of Site 6 and 1078 ± 2.359 mg/L in second year of Site 6 during the study period (Tables 5.16 and 5.17). Turbidity in water is caused by suspended and colloidal matter such as clay, silts, finely divided organic and inorganic matter, plankton and other microscopic organisms. Turbidity diffuses sunlight and slows photosynthesis. Plants begin to die, reducing the amount of dissolved oxygen and increasing the acidity by producing carbonic acid and lowers the pH level. Both of these effects harm aquatic animals. Turbidity raises water temperature because the suspended particles absorb the sun's heat. Warmer water holds less oxygen, thus increasing the effects of reduced photosynthesis. Some eggs and larval stages of aquatic animals may not adjust well to the

warmer water. Highly turbid water can clog the gills of fish, stunt their growth and decrease their resistance to diseases.

The organic materials that may cause turbidity can also serve as breeding grounds for pathogenic bacteria. The study revealed that turbidity was higher in rainy and lower in summer seasons in both sites. There was high fluctuation of turbidity during rainy season due to flooding (Tables 5.14, 5.16 and 5.17).

Different physico-chemical factors of water of six water bodies were studied for two years. Out of six water bodies EUS outbreaks were recorded in three fish farms (Baidya fish Farm, Babiya Birta Fish Farm and Tarahara Fish Farm from 2008-2015. Generally the outbreak of EUS takes place in the month of December and persists upto March. No outbreak of the disease was found in three water bodies viz. Betana Wetland, Singhia River and Budhi River. Results showed that prior to the outbreak of the disease there were significant ($p<0.01$, $p<0.05$) declining trends of two factors e.g. total alkalinity and total hardness of water of the three fish farms and the factors remained low for few months (Chapter V; Sec.5.1; Tables 5.1 to5.9; Figs.5.8, 5.9, 5.11, 5.12, 5.21, 5.22, 5.25, 5.26,5.34,5.35,5.38,5.39). Declining trends of total alkalinity and total hardness were also noticed in other water bodies where no outbreak of disease was found (Chapter V; Sec.5.1; Tables 5.10 to 5.18; Figs.5.47,5.48,5.51,5.52,5.61,5.62,5.65,5.66,5.75,5.76,5.79,5.80).

Fall of surface water temperature was noticed in all water bodies studied during the onset of the disease. Water temperature remained below 30ºC most of the time of the year and only for a short period of time it goes above 30ºC (Tables 5.1, 5.2, 5.4, 5.5, 5.7, 5.8). It appeared that this condition is favourable for the growth of bacteria and fungus.

Chinabut *et al.* (1995); Lilley *et al.*(2002) mentioned that generally, EUS occurs when the water temperatures are comparatively low either because of a sudden drop in temperature associated with massive rainfalls or in the cold season of the year. EUS has been observed in a wide temperature range: the disease occurred in freshwater ponds in Louisiana in the winter months at water temperatures from 10 to 15ºC (Hawke *et al*., 2003) and in fish pond in the Philippines at temperatures as high as 33ºC (Bondad-Reantaso *et al.*, 1992).

The disease is not a simple result of contact between host, pathogen and environment (Snieszko, 1974).Sub lethal changes in water quality may stress sufficiently to predispose fishes to infectious diseases (Wedemeyer, 1974).

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 outbreak occurred in cyclic manner when the temperature falls after heavy rainfall. The incidence of disease outbreak was high in water of low alkalinity and hardness (Jhingran, 1990; Kar, *et al.,* 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 that there were variations in temperature, chloride, rainfall and hardness of water at the time of EUS outbreak in Philippines. Das and Das (1993) reported that in West Bengal, the disease outbreak occurs after the monsoon when the water temperature falls and incidence is more in confined waters (10-55%) than in rivers (4-15%). 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. Localized EUS outbreaks were reported after a rainfall and were associated with pH values between 5 and 6.3. Lumanlan-Mayo *et al.* (1996) suggested from the field and laboratory experiments that low water temperature <30ºC 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 immune suppression at low temperature. Ahmed and Hoque (1998) observed that the 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. Roy and Pal (2003) showed that the outbreak of EUS occurred in three ponds of different areas of Jalpaigudi district, West Bengal, India when dissolved oxygen content, hardness and total alkalinity of pond water remained low. So from the above discussion and from the results of the experiment conducted for two years from November, 2008 to October, 2010 on the water parameters of the water bodies of the diseased prone areas. Disease outbreak occurred only in the ponds where fishes are cultured. It appeared that apart from the declining temperature, total alkalinity and hardness of water cultural practices may have some roles in EUS outbreaks.

During the entire study period 444 EUS affected fish of different species were collected (Table 5.35). The ulcers were categorized into three major types i.e., superficial, moderate and severe depending on the nature of ulcer (Chapter-V, Sec. 5.2, Figs. 5.81a and b to 5.88a and b, 5.89 to 5.92). In natural condition, the present study showed that order of susceptibility was about 60% *Cirrhinus mrigala*, 30% *Labeo bata / L. rohita*, 7% *Catla catla*, 3% *Channa striatus*, *Puntius* sp., *Heteropneustes fossilis*, *Mystus tengara* and *Clarias batrachus* among a total 444 affected fishes. However, only small body lesion was found in *C. carpio* which was originally known as resistant to EUS (Bondad-Reantasho *et al.*, 1992). So the further confirmatory test should be conducted in *Cyprinus*. EUS appeared prevalently in February, March-April and October-November in Nepal.

The histopathological observations in ulcer of different fish showed loss of epidermis, granulomatous changes and necrosis of the muscle. In the present experiment, several species of naturally infected fish and three types of ulcers i. early stage (single or multiple red spots on body surface) (Fig.5.90), ii. moderate type (erosion of epidermis) (Figs.5.83, 5.89) and iii. advanced type (deep and necrotic ulcer with occasional haemmorhages) (Figs.5.81 a and b, 5.85 a and b) were recorded and observed histopathologically. The ulcer of all that fish showed the presence of non-septate invasive fungal hyphae (Sec.5.3) which were stained black with

Grocott's methenamine silver stain (GMS) (Figs.5.101,5.102,5.107,5.108,5.113,5.114,5.117, 5.121) and pink with PAS stain (Figs.5.104,5.118,5.122). 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 underlying musculature (Fig.5.122). Sections of the liver of infected fish showed the presence of the hepatocytes vacuolation and infiltration of blood capillaries (Fig.5.109). Sections of kidney showed tubular vacuolation and necrosis in some regions (Figs. 5.111, 5.112).

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) categorized 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 5 types depending on clinical patterns: Type I, Type-II, Type-III, Type-IV and Type-V.

Pal and Pradhan (1990) collected 129 *Anabas tesudineus*, 16 *Heteropneustes fossilis* and 11 *Clarias batrachus* from different affected areas of North Bengal. They observed that the symptoms of the disease first appeared as a red spot in scale less fish. Gradually the red spot increased in size and ulcer developed in the affected area. Ultimately the underlying muscle layer became affected and occasionally the ulcer became deep and necrotic. In scaly fish, red spot appeared in some regions of the body. Ultimately the scales were sloughed and the ulcer became deep and necrotic. Fins were also affected in both cases.

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 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 hematopoietic tissue showed proliferation of macrophage cells which indicated the inflammatory response. Qureshi *et al.* (1995) found that the epidermis of the infected *C. batrachus* was destroyed completely and wherever it existed showed spongiosis. The dermis lost its original compactness.

Pradhan *et al.* (1991) reported infection of different stages of development in *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala.* Mohan and Shankar (1995) reported the presence of fungal hyphae with severe necrosis of the dermis and epidermis which were surrounded by one to several layers of macrophages and infested by fibroblasts. Cruz-Lacierda and Shariff (1996) reported the association of highly invasive broad branched aseptate fungal hyphae.

Fungal hyphae invade the muscle in all directions from the centre of the dermal tissue. The advanced stage ulcer 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 (Viswanath *et al.,* 1998). But in this experiment no fungal invasion in liver, kidney and spleen was found.

In the present study, twenty three bacterial isolates were collected from the ulcers of six different EUS affected fish. The morphological features and biochemical profiles of eighteen isolates suggested that they were motile, non-spore forming, glucose fermenting, gram-negative bacilli. They were straight rods, grown in agar with 0% but not in 6% NaCl and reduced nitrate to nitrite (Tables 5.36, 5.37, 5.38, 5.39, 5.40 and 5.41). Thus, they all belonged to the genus *Aeromonas* (Popoff, 1984). Among the eighteen isolates, fourteen (Cm_1,Cm_3,Cs_1,P_1,P_3,Lb_2,Lb_3 ,Cc_1,Cc_2,Cc_3,Mt_1,Mt_2,Mt_3 and Mt_4) of them were positive to esculin hydrolysis test, sensitive to antibiotic cephalothin, produced gas from glucose and acid from arabinose (Carnanhan *et al*., 1991). These bacteria were positive to lysine decarboxylase and arginine dehydrolase test but negative to ornithine decarboxylase test, produced acid from mannitol and sucrose and gave a positive to VP test (Abbott *et al.*, 1992). Thus, these bacteria were regarded as *Aeromonas hydrophila*. The isolate Lb_4 was negative to esculin hydrolysis test, positive to indole test, produced acid from sucrose and gave a positive VP test (Carnanhan *et al*., 1991). It was tested positive to arginine dihydrolase, lysine decarboxylase and negative to ornithine decarboxylase, produced acid from arabinose and mannitol (Abbott *et al.*, 1992). Therefore, it belonged to *Aeromonas veronii biovar sobria.* The isolate Cm_4 and Cs_2 and Cs_3 (Tables 5.36, 5.38) were tested positive to esculin hydrolysis but could not produce gas from glucose (Carnanhan *et al*., 1991). It was tested positive to arginine dihydrolase, negative to lysine decarboxylase and

ornithine decarboxylase, gave a negative VP test and produced acid from arabinose, mannitol and sucrose (Abbott *et al.*, 1992). Thus these were identified as *Aeromonas caviae* (Fig. 5.127).

The morphological features and bio-chemical profiles of the isolated pathogenic bacteria, Cc_4 and Lb_1 (Tables 5.37,5.41, 5.42; Fig. 5.129) revealed that these bacteria were gram negative, motile, catalase positive, utilized glucose oxidatively and produced yellowish green pigment in King's B medium. Thus they belonged to the genus Pseudomonas (Stanier *et al.*, 1966, Palleroni, 1984).

The biochemical profile of Cm_2 and P_4 (Tables 5.36, 5.39, 5.42) showed that these bacterial isolates belonged to the genus Micrococcus (Fig.5.128). These differed from the genus *Staphylococcus* and *Streptococcus* with respect to the breakdown of glucose. As these isolates utilized glucose oxidatively and produced a yellowish pigment, Cm_2 and P_4 resembled Micrococcus (Fig.5.128). The sphere-shaped bacteria were 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 25-37ºC and non-pathogenic. From the morphological and biochemical profile, P_2 isolate (Tables 5.40, 5.42) resembled Moraxella.

The genus Neisseria is also catalase and oxidase positive but morphologically they are coccoid (Vedros, 1984).

Fourteen *Aeromonas hydrophila* (Fig.5.130) were isolated from infected fishes e.g. *C. mrigala, C. catla, C. striata, Puntius* sp., *Mystus tengara* and *Labeo bata* and all are pathogenic. Three *Aeromonas caviae* were isolated from ulcers of *Cirrhinus mrigala* and *Channa striata* and one *A. veronii biovar sobria* was isolated from ulcer of *Labeo bata* .These are also pathogenic.

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* (Hosina, 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 exophthalamus and subcutaneous ulceration. Rahim *et al.* (1985) isolated *A. hydrophila* from the wounds of five species of fishes even before the outbreak of EUS in Bangladesh. Okpokwasili and Okpokwasili (1994) found that *Pseudomonas* spp. and *A. hydrophila* isolated from the brown patch disease of tilapia were more virulent to tilapia fingerlings when infected by mix culture than *A. hydrophila* or *Pseudomonas* spp. alone. Esteve *et al.* (1993) isolated *A. hydrophila* and *A. jandaei* from diseased European eel from an eel farm in Spain which caused ulcer disease by intraperitoneal injection (LD50 dose: 105.4 to 107.5 cfu / fish) and also by bath exposure to 107 to 108 cfu / fish 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 from 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 liquefaciens, 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. Reinfection studies in carp and goldfish showed that percentage 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 micro organisms, *A. punctata*, *P. fluorescens* and a virus may be involved in this disease which manifests itself in several forms (Schäperclaus, 1969).

Two pathogenic *Pseudomonas* sp. were isolated from ulcer of *Catla catla* and *Labeo bata* .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 hemorrhage 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 the 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 were able to induce the same clinical signs as those observed during disease outbreak by reinfection treats.

Pseudomonas fluorescens has been reported to cause hemorrhagic septicemia in European eel, *A. vulgaris* (Andre *et al.*, 1970) and 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 (1986) reported induction of ulcer in *A. testudineus* by mixed culture of two bacteria, one fluorescent Pseudomonad and another coccus, *M. varians*.

Different species of Pseudomonas had been reported to be the causative agent of various fish diseases, throughout the world. *P. anguilliseptica* which caused hemorrhage 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.

Llobera 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 people's Democratic Republic, Malaysia, 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 McGarey *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). Likewise one aeromonad (*A. hydrophila*), two pseudomonads and one coccus (*Micrococcus varians*) were isolated from the ulcer tissues of *C. mrigala* (Pradhan *et al*., 1991).

Two *Micrococcus* sp. were isolated from ulcer of *Cirrhinus mrigala* and *Puntius* sp., one *Moraxella* sp. was isolated from ulcer of *Puntius* sp. Two *Micrococus* sp. and one *Moraxella* sp. are non-pathogenic.

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), Mc Garey *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 from 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 affected fish of Karnataka, India. Aeromonads and Pseudomonas, 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; Leano *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 affected fish out of which *Pseudomonas* sp. and *Aeromonas* sp. was 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 6 strains of Aeromonads and Pseudomonads were pathogenic and the rest were non- pathogenic. They showed that four Aeromonads out of 16 strains induced ulcers in healthy *A. testudineus* when injected intramuscularly. Several workers have isolated different types of bacteria such as *Aeromonas* sp., *Micrococcus* sp., *Acinetobacter* sp. and *Streptococcus* sp. from the ulcer of affected fishes (Kar, 1999, 2000; Das *et al*. 1990; 2001).

Nile tilapia (*Oreochromis niloticus*) was found to be resistant to EUS (Ahmed and Rab, 1995). The relative susceptibilities 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*. 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. EUS affected fishes often die because of bacterial septicemia caused by pathogenic aeromonads entering the circulation through haemorrhagic dermal lesions that characterises the disease (Pal and Pradhan ,1990; Rahman *et al.*, 2002).

Mastan and Qureshi (2001) reported that 17 species of bacteria were isolated from the investigated water bodies and EUS affected fishes. The species of bacteria isolated from fishes are common to those isolated from water. Experimental infection trials conducted suggested that *Aeromonas hydrophila* in association with *Pseudomonas fluorescens* may be playing the role of primary etiological agent in producing EUS in fishes. Das *et al*. (2009) found drug resistance and plasmid mediated transferability in 15 Aeromonas isolates collected from the ulcers of epizootic ulcerative syndrome (EUS) affected fishes *Catla catla, Cirrhinus mrigala* and *Puntius* sp.. Disc diffusion assay showed that all the strains were resistant to ampicillin and sensitive to streptomycin.

From the experimental work, it is found that out of 23 bacterial strains isolated from infected fish fourteen *Aeromonas hydrophila,* three *Aeromonas caviae*, one *A. veronii biovar sobria* and two

Pseudomonas sp. are pathogenic. Two *Micrococus* sp. and one *Moraxella* sp. are non-pathogenic.

An aseptate fungus was isolated from EUS affected *C. mrigala* in 2013. The fungus formed sporangia at the hyphal tip not wider than hyphae in GPY medium and ball of spores was noticed at the tip of the sporangium. The fungus did not grow at 37ºC. From these characteristics, it appeared that the isolated fungus was *Aphanomyces* sp.. Experimentally, the fungal zoospores induced ulcer in healthy *H. fossilis* (Fig.5.142) and produced typical granulomas in the dermis and underlying musculature (Fig.5.143). Presence of the fungus was also detected in the sections of the ulcer of the experimental fish (Fig. 5.144).

Roberts *et al.* (1993) reported that the survey of fish affected with epizootic ulcerative syndrome in different countries throughout South and Southeast Asia showed constantly presence of morphologically typical fungus within the lesion. When the mycelium from pathogenic isolates of *Aphanomyces* sp. was placed below the dermis of healthy fish, it caused an inflammatory response and spread 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 31ºC. 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 26ºC and 31ºC while fish kept in 19ºC developed a severe invasive myonecrosis with limited macrophage response. From 14 to 28 days, post-infection healing became well established at 26ºC and 31ºC 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 19ºC and all such fish succumbed by this time. The mortalities in the fish kept at 19 ºC were considerably higher 40% than in the group of fish kept at 26ºC and 31ºC (12%). The findings explain the mortalities from EUS occurring when water temperatures are low.

Several workers reported the association of *Aphanomyces* sp. with fish disease. Srivastava (1979) reported *Aphanomyces* sp. infection in *Clarias batrachus*, Uttar Pradesh, India in 1973, long before the breakout of EUS in India. Noga (1994) reported that ulcerative mycosis (UM) in USA was caused by *Aphanomyces* sp. Blazer *et al.*(1999) reported that *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 harboured a deeply invasive, highly pathogenic fungus now to be *A. invadans*.

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* (Willoughby *et al.*,1994). The protein banding profiles (Callinan *et al.* 1995b; Lilley *et al.*, 1997b), growth characteristic (Lilley and Roberts,1997) and genetic fingerprinting techniques (Lilley *et al*. 1997a) showed that various *Aphanomyces* sp. isolated by various workers were very same and *A. invadaris* was renamed to *A. invadans* (David and Kirk, 1997). 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.

Saha (1998) showed that the mortality rate of *A. testudineus* inoculated with isolated bacteria R1, R2 and R3 were 39%, 40% and 40% respectively.

In contrary, Afzali *et al.* (2013) isolated and identified fresh water fungi species from the Malaysian natural water bodies and fish farms and to examine the pathogenicity of the isolates as a confirmative identification tool for epizootic ulcerative syndrome (EUS) outbreak in Selangor state, Malaysia. For that 165 water samples and 62 infected fish collected from 12 stations were tested in which 35 and 24 samples were found to be positive for fungi contamination and /or infection, respectively. The isolates were morphologically characterized; from 59 isolates, 32 were identified as saprolegina, 21 as Achlya and six as *Aphanomyces* species. Experimental infection was carried out by intramuscularly injection of the *Aphanomyces* spp. isolates to the Malaysian moonlight gourami (*Trichogaster microlepis*), where no mortality and no signs of EUS were observed in the fish groups. Histopathology test also revealed no signs of damage in the skin, muscles and other tissues following infection with the isolates indicating that all the *Aphanomyces* isolates were non-pathogenic.

The mortality rate of fish in the outbreak of the disease was much more than that of the experimentally inoculated fish by zoospores of isolated fungus. The section of ulcer of fungal

zoospore inoculated *H. fossilis* showed typical granulomatous changes very similar to the naturally infected fish (Figs.5.142, 5.143, 5.144). *Aeromonas hydrophila* isolated from ulcers of different infected fishes induced ulcers in experimental fish (Figs.5.135, 5.136,5.137).

Although lowering of water temperature,total alkalinity and total hardness was supposed to be helpful to outbreak the EUS disease it is still unclear because in similar physico-chemical conditions outbreak of the disease occurred in some areas (S1,S2 and S3) but not occurred in other areas (S4,S5 and S6). Some other factors to be considered in future research work. The intense rearing of fish through high stocking densities, use of artificial feed and fertilizer, application of chemotherapeutic agents have not only led to increase the large scale fish production throughout the world but also created conditions leading to the physiological stress and an increasing risk of disease outbreak (Pillay, 1996; McLean, 1996).

Other fish diseases abdominal dropsy, haemorrhagic septicaemia of carps, fin rot and gas bubble disease were recorded as minor diseases of fishes.

In case of other bacterial fish disease, Mukherjee (1992) attributed cases of dropsy to *A. hydrophila* in conjuction with malnutrion. Scientists at CIFA have been reported that the fin and tail rot in young fish are due to a mixed infection of *A. hydrophila* and *Pseudomonas florescens.* They also elucidated the role of *A. hydrophila* in dropsy fin and tail rot and more elaborately in epizootic ulcerative syndrome (Mukherjee, 1996).

From the above discussion and results of the experiments (Figs.5.1,5.2,5.8,5.9 5.11-5.13,5.15, 5.21,5.22,5.24, 5.26, 5.28, 5.34, 5.35, 5.37- 5.39, 5.41, 5.47,5.48, 5.50-5.52, 5.54, 5.61,5.62,5.64-5.66,5.68,5.75,5.76,5.78-5.80; Figs. 5.81a and b to 5.88a and b ; Tables 5.36 to 5.42), it can be concluded that both bacteria and fungus *Aphanomyces* sp. play important roles when temperature remains low in winter months in the appearance of EUS (Epizootic Ulcerative Syndrome) and it is the major fish disease but other fish diseases **(**abdominal dropsy, haemorrhagic septicaemia of carps, fin rot and gas bubble disease) are minor fish diseases in eastern Nepal.