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Role of physicochemical factors of pond water on the outbreak of epizootic ulcerative syndrome and histopathology of affected fishes from eastern Nepal

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Abstract
EUS/MG/RSD/UM/EGA has been considered the most serious disease of fish and caused heavy loss since its initial outbreaks (February 1989) generally occurring during November to March in Nepal. Three fish farms - Baidya Tankisinwari, Babia Birta and Tarahara were selected as disease prone areas of Eastern Nepal. Water analysis was conducted monthly for two years (November 2008 to October 2010). Histological slides were prepared from infected fish tissues and stained with H E, GMS and PAS observed by microscope. Smear of slightly ulcerated tissue was stained with cotton blue for fungi. This work evaluates the prevalence of epizootics, predisposing environmental factors, route of causative agent into fishes and biosecurity.

High fluctuation of water temperature, low pH, DO, decreasing TA and TH suppress the immunity of fishes and easily attacked by *A. invadans* from contaminated water outbreak the EUS. In Tarahara pond though the condition was almost similar but EUS did not appear which clearly showed that such condition may not always develop the EUS. Among collecteted fishes about 60% *Cirrhina mrigala 30% Labeo bata/L. rohita*,10% *Channa* sp., *Puntius* sp., *Mystus* sp., *Catla catla and Heteropneustes fossilis* were EUS affected in which pinhead-sized, red spots on the body surface, head and fin, caudal peduncle, operculum with no visible ulcers but acute dermatitis forming rosacea ultimately the muscle ulcers became deep and necrotic. Histological slides revealed granulomatous, Aphanomyces filaments in muscle, vacuolation, necrosis in the liver and kidney led to death. Farmers reduce the risk of heavy loss from EUS by adopting treatment and preventive measure.

Keywords: Physicochemical, predisposing factors, EUS, fish, *Aphanomyces invadans*, histopathology.

1. Introduction
Epizootic ulcerative syndrome (EUS)/mycotic granulomatosis (MG)/red spot disease (RDS)/ulcerative mycosis (UM), is caused by the oomycete *Aphanomyces invadans* as primary fish pathogen. Scientists proposed that EUS should be named as epizootic granulomatous aphanomycosis (EGA) [4]. The causative agent was *Aphanomyces invaderis* [28] which can develop significant ulceration of the skin, necrosis of muscle and propagates to adjacent structures and mostly leading to mortality. It was firstly described in cultured ayu (*Plecoglossus altivelis*) in Japan in 1971 [8]. Parasites and rhabdo viruses have also been associated with particular outbreaks, and secondary gram-negative bacteria invariably infect EUS lesions.

Susceptible fish, infective forms of the fungus and suitable environmental conditions leads to outbreak of EUS. Ahmed and Rab (1995) [1] associated EUS outbreaks in Bangladesh with farming of susceptible fish species in ponds which had previously been ruined, or treated with piscicides to remove predators and other undesirable fish prior to stocking. They found that the fungus must have survived in these ponds, either within surviving infected fish or in the environment, possibly as an encysted spore. Flooding also caused the spread of EUS in Bangladesh and Pakistan [10].

EUS outbreaks in estuarine fish are often associated with recent acidified runoff from acid sulfate soil areas. Possibly soil or sediment characteristics influence outbreak in freshwater ponds. Sediments at many outbreak sites were slightly acidic and had low calcium content [12]. An association between EUS outbreaks and ponds having reddish sandy soils and relatively high turbidity may have been stressful to fish [1]. A number of predisposing factors leading to infection have been identified. Heavy rainfall and soils which are either naturally acidic or

disturbed by agriculture or residential development may lead to a lowering of water pH [4, 7]. Disease often manifests when water temperatures drop, possibly as a consequence of a retarded immune response in the fish and increase susceptibility to infection with *A. invadans* [4] and may partly explain the relatively high seasonal prevalence of infection in fish of floodplains.

Spread of *A. invadans* occurs via zoospores in the water. Secondary zoospores enter the skin following a breach of the epidermal barrier by physical or environmental causes [4]. The zoospores germinate and hyphae invade the skin and musculature, causing a focal necrotizing granulomatous dermatitis and myositis. Aphanomyces was long regarded as a fungus but the group Oomycete has recently been classified with diatoms and brown algae in a group stramenopiles or Chromista. A group of filamentous, unicellular heterokonts or stramenopiles physically resembling fungi; they are microscopic, absorptive organisms that reproduce both sexually and asexually and are composed of mycelia [9].

EUS has been considered the most serious disease affecting freshwater fish since its initial outbreaks in February 1989 from eastern part of Nepal [22, 23]. EUS occurs November to March in different parts of the country every year but no considerable research has been done yet. In the present study, an attempt has been made to find out the role of the physicochemical parameters of water to outbreak the EUS and isolation and propagation of pathogenic oomycete from the ulcers of EUS affected fish in Eastern Nepal. The objectives are to evaluate: i. The prevalence of epizootic 2009 onwards and environmental factors associated with it. ii. The route of introduction of *A. invadans* into fishes in eastern Nepal. iii. Recommendation for future research and biosecurity.

2. Materials and Methods

To detect the presence of areas of trauma, ulcers, abscesses, loss of color and parasites, the natural openings and skin. For external appearance or autopsy of diseased fishes, the mouth, nasal orifices, head and opercula were observed with naked eyes and magnifying glass for deformities or moldy part.

2.1 Methodology

Study area

Baidya fish farm Tankisinwari (S1), Babia Birta fish farm (S2) and Tarahara fish farm (S3) were selected in the disease prone areas of Eastern Nepal. Water analysis was conducted at monthly intervals for a period of two years.

Site -1(s1) **Site-2(s2)** **Site-3 (s3)**

2.2 Physico-chemical parameters of water samples

Temperature, pH and Turbidity were estimated on the site by glass thermometer, Hanna's pocket pH meter and turbidity meter respectively.

All other physico-chemical analysis of water like dissolved oxygen (DO), biological oxygen demand (BOD), free carbon dioxide (free CO_2), chloride ions (Cl^-), ammonia (NH_3), total alkalinity and total hardness were determined following standard methods [3, 25].

2.3 Statistical analysis

Standard deviation, correlation coefficient were calculated by using Microsoft excel statistical function of computer software. The correlation coefficient between different variables is calculated and their significance difference was tested using SPSS-20.

2.4 Collection and Diagnosis of diseased fish

Infected fish showing body lesions and fin rot, believed to be the result of injury and subsequent infection were collected from the selected sites and brought to the laboratory in live condition. The fish were maintained in glass aquaria measuring 90 cm X 35 cmX 35 cm. For the preparation of histological slides after dissecting diseased fish tissues, immediately fixed in 10% formalin. The study was conducted from November 2008 to October 2010.

2.5 Methodology for pathogenesis study

Fungal isolation and culture

Fungi were isolated from the infected fish following the method of Willoughby and Roberts, 1994 and Lilley *et al.* 1998 [29, 10] and cultures of the same were maintained by aseptically transferring the fungus to freshly prepared fungal media. Sporulation of fungus was done by growing the mycelium in GPY broth. Code names were assigned to each of the isolate until identification.

2.6 Microscopic and histopathological examination

A portion of the ulcer tissue was taken and smeared in a clean glass slide. The smear stained with cotton blue and observed under the microscope. The fungal hyphae and sporangium from the pure culture were stained and observed with the microscope. Diseased tissues, liver and kidney were taken out after dissected from infected fish and fixed in Bouin's fixative. Tissue sections of 6 µ were cut from the fixed samples using the standard microtome technique. Staining of sections was done with haematoxylin-eosin, Grocott hexamine silver stain and Periodic Acid Schiff's stain. Histopathological abnormalities and presence of fungus was examined using the microscope.

3. Results and D iscussion

Infected fishes

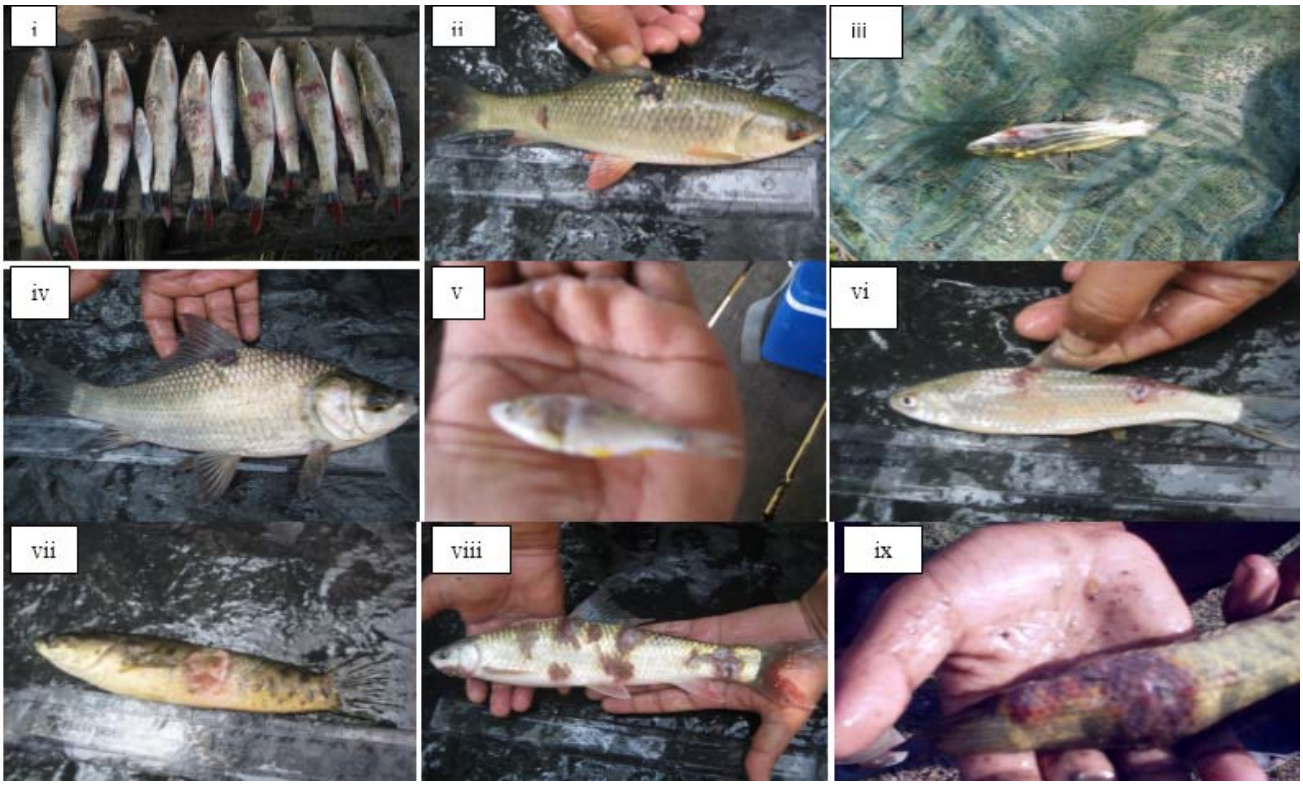


Fig 1: i. Affected fishes ii. *Labeo rohita* iii. *Mystus* sp. iv. *Catla catla* v. *Puntius* sp. vi. *Labeo bata* vii/ix. Channa *striatus* viii. *Chirinus mrigala*.

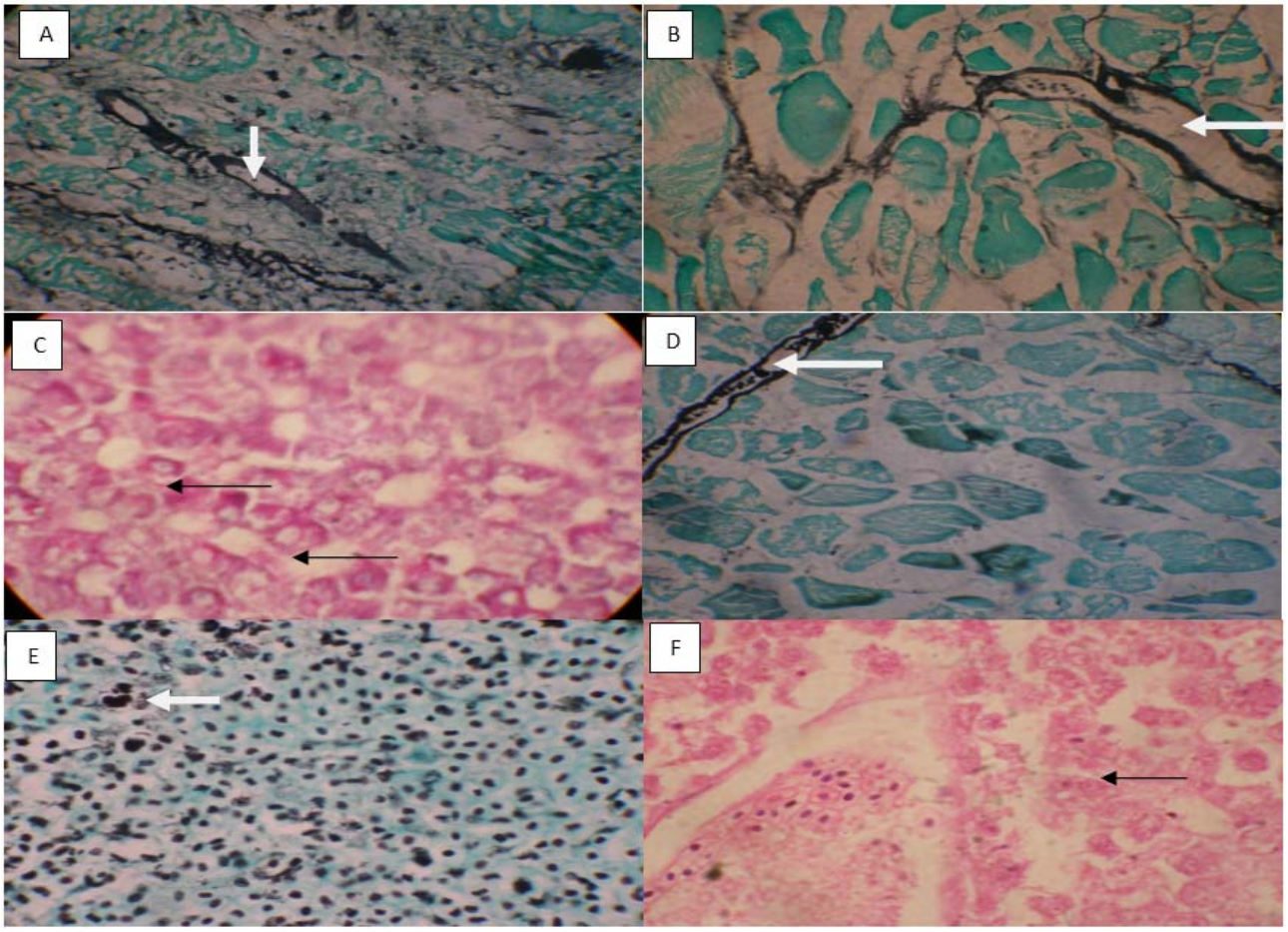
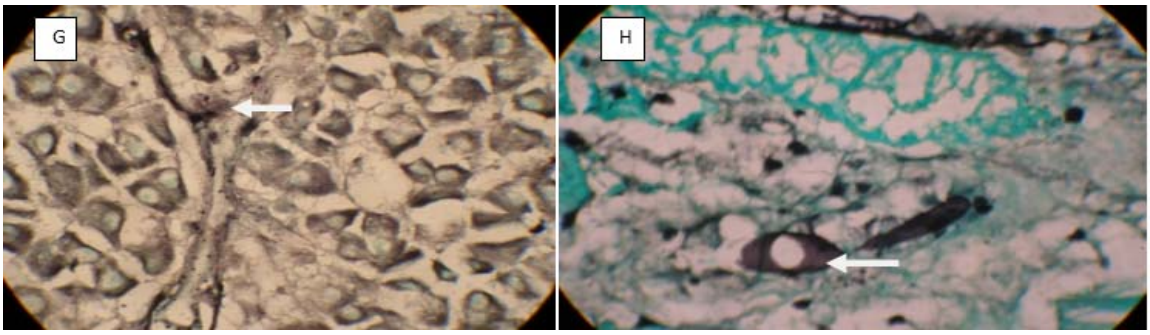


Fig 2: A. *Cirrhinus mrigala* muscle T.S. showing Aphanomyces invadans filament. B. *Labeo bata* muscle T.S. showing Aphanomyces invadans filament C. *Cirrhinus mrigala* liver T.S. showing necrosis and vacuolation. D. *Catla catla* muscle T.S. showing *Aphanomyces invadans* filament. E. *Labeo bata* T.S. liver F. *Labeo bata* T.S. Kidney (H-Ex400).



G. *Catla catla* muscle (PASx400) H. *Catla catla* T.S. muscle showing *A. invadans* filament.

Table 1: Shows physicochemical parameters of Baidya fish farm (S1) during Nov. 2008 – Oct. 2009*(Mean±S.D. N=5).*

Parameters	Months											
Site1 – I Yr.	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept.	October
W Temp. 0 C	21.12	17.05	18.2	22.15	24.35	29	28.52	28.17	25.07	28.17	29	26.07
	±0.095	±0.550	±0.216	±0.173	±0.506	±0.216	±0.170	±0.150	±0.170	±0.150	±0.320	±0.170
pH	7.62	8.17	8.12	8.3	8.2	6.22	6.5	7.32	6.37	6.72	7.25	7.82
	±0.05	±0.150	±0.120	±0.170	±0.170	±0.309	±0.081	±0.095	±0.309	±0.095	±0.129	±0.098
DO mg/L	4.8	5.88	6.27	7.28	7.16	7.83	7.04	7.47	7.04	5.52	6.25	6.52
	±0.335	±0.078	±0.170	±0.022	±0.035	±0.297	±0.009	±.032	±0.009	±0.083	±0.127	±0.090
TA	137.3	97.76	133.12	156	187.2	198.2	208	166.25	158.2	110.75	101.2	128.5
mg/L	±0.208	±0.721	±0.095	±1.173	±1.676	±0.559	±0.452	±8.957	±0.843	±0.208	±0.543	±0.368
T H	118.4	122.4	105.2	107.6	144.6	123.6	118.3	90.2	90.8	82.19	101.5	106.1
mg/L	±1.25	±0.573	±0.08	±0.660	±0.463	±0.657	±1.25	±0.095	±0.028	±0.679	±0.164	±0.121

Table 2: Shows physicochemical parameters of Baidya fish farm (S1) during Nov. 2009 – Oct. 2010*(Mean±S.D. N=5).*

Parameters	Months											
Site1 – IIYr.	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept.	October
WTemp.	25.5	19.1	17.3	22.2	28.5	29.5	28.5	29.5	30.2	30.3	31.4	29.2
0 C	±0.535	±0.273	±0.526	±0.216	±0.415	±0.082	±0.415	±0.082	±0.216	±0.051	±0.327	±0.216
pH	8.3	8.9	8.2	8.2	7.8	8.3	9.2	8.8	8.5	8.9	9.1	8.8
	±0.170	±0.097	±0.095	±0.095	±0.221	±0.095	±0.320	±0.096	±0.081	±0.097	±0.150	±0.096
DO mg/L	10.17	8.83	7.34	6.67	6.71	2.7	8.64	6.67	6.69	6.61	9.31	10.73
	±0.221	±0.521	±0.231	±0.452	±145	±0.248	±0.215	±0.046	±0.118	±0.340	±0.561	± 0.258
TA	109.89	104	150	243.6	162.5	154	154	121.9	92	101.2	99	83.6
mg/L	±0.891	±0.865	±1.02	±0.521	±0.756	±0.884	±1.062	±0.645	±0.766	±0.443	±0.355	±0.325
T H	91.02	49.5	130.56	130.68	132.66	126.72	126.72	81.18	75.24	77.22	79.2	73.26
mg/L	±1.035	±0.463	±0.647	±0.751	±0.463	±0.458	±0.095	±0.844	±0.363	±0.537	±0.237	±0.572

Table 3: Shows physicochemical parameters of Babia birta fish farm (S2) during Nov. 2008 – Oct. 2009*(Mean±S.D., N=5).*

Site2 – IYr.	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept.	October
WTemp.	20	17	18	21	23	30	28	29	29	29	29.5	25
0 C	±0.374	±0.452	±0.215	±0.336	±0.223	±0.526	±0.456	±0.126	±0.456	±0.371	±0.217	±0.275
pH	8.8	8.1	8.7	7.4	6.8	6.6	7.3	7.2	6.6	7.4	8.3	8.7
	±0.24	±0.212	±0.325	±0.216	±0.332	±0.315	±0.168	±0.256	±0.122	±0.345	±0.470	±0.335
DO mg/L	7.67	4.96	7.67	7.44	7.83	6.65	6.26	6.65	6.65	6.88	6.16	7.66
	±0.223	±0.089	±0.342	±0.421	±0.325	±0.210	±0.167	±0.208	±0.097	±0.275	±0.551	±0.345
TA	80.36	67.68	108.16	105.04	124.8	115.4	135.3	135.2	114.4	95.94	69.3	79.8
mg/L	±0.563	±0.32	±0.336	±0.345	±0.442	±0.642	±0.453	±0.351	±0.667	±0.655	±0.671	±0.539
T H	77.52	91.8	82	80.2	90.66	80.6	76	92	94	86.4	84.24	69.36
mg/L	±0.661	±0.546	±0.711	±0.534	±0.477	±0.576	±0.635	±0.895	±0.932	±0.655	±0.563	±0.736

Table 4: Shows physicochemical parameters of Babia birta fish farm (S2) during Nov. 2009 – Oct. 2010 *(Mean±S.D. N=5).*

Site2 – IIYr.	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept.	October
WTemp.	24	19	17.5	20	27	29	29	29	30	30	31	28
0 C	±0.219	±0.231	±0.315	±0.355	±0.218	±0,332	±0.273	±0.344	±0.265	±0.556	±0.342	±0.213
pH	7.5	8.3	7.8	8.7	8.5	7.3	8.1	7.6	8.5	7.9	8.2	7.5
	±0.231	±0.175	±0.114	±0.211	±0.253	±0.231	±0.223	±0.098	±0.347	±0.216	±0.310	±0.128
DO mg/L	5.56	7.14	7.86	9.71	5.94	3.8	5.37	4.94	5.82	6.17	6.2	6.3
	±0.164	±0.344	±0.231	±0.257	±0.221	±0.321	±0.211	±0.225	±0.097	±0.203	±0.242	±0.313
TA	141.6	128	100	151.2	82.5	110	176	108.1	101.2	99	112.2	112.2
mg/L	±0.655	±0.438	±0.677	±0.757	±0.486	±0.539	±0.875	±0.459	±0.443	±0.376	±0.445	±0.558
T H	116.8	63.36	99.96	87.12	81.18	104.94	102.86	99	85.15	83.16	93.06	99
mg/L	±0.996	±0.765	±0.457	±0.540	±0.412	±0.345	±0.431	±0.330	±0.243	±0.289	±0.376	±0.435

Table 5: Shows physicochemical parameters of Tarahara fish farm (S3) during Nov. 2008 – Oct. 2009*(Mean±S.D. N=5).*

Site3 – IYr.	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept.	October
WTemp.	18.75	15.3	18.75	21.5	23.57	29.12	27.07	27.45	27.07	27.12	27.25	25.27
0 C	±0.228	±0.489	±0.288	±0.408	±0.434	±0.275	±0.25	±0.42	±0.25	±0.275	±0.645	±0.499
pH	7.9	8.05	8.62	8.12	7.325	6.67	8.12	8.2	7.05	7.12	8.2	7.62
	±0.089	±0.129	±0.095	0.095±	±0.095	±0.125	±0.629	±0.081	±0.057	±0.275	±0.216	±0.478
DO mg/L	5.71	5.84	8.92	8.61	7.86	8.1	7.04	7.83	8.93	4.86	5.45	5.75
	±0.335	±0.079	±0.221	±0.115	±0.354	±0.127	±0.225	±0.009	±0.553	±0.079	±0.245	±0.365
TA	147.96	128.7	202.5	194.95	176.82	157.7	167.12	125.62	135.8	118.07	103.4	133.02
mg/L	±1.860	±1.112	±5.802	±1.962	±1.189	±0.877	±0.689	±0.805	±0.585	±0.449	±0.469	±0.694
T H	138.72	157.1	164.4	148.6	146.14	101.2	96.32	91.2	83.6	92.88	108.25	118.23
mg/L	±2.125	±1.325	±1.478	±1.036	±0.985	±0.776	±1.745	±1.558	±0.998	±0.756	±0.955	±0.779

Table 6: Shows physicochemical parameters of Tarahara fish farm (S3) during Nov. 2009 – Oct. 2010 *(Mean±S.D. N=5).*

Site3 – IIYr.	Nov	Dec	Jan	Feb	March	April	May	June	July	August	Sept.	October
WTemp.	21.26	17.31	17.45	19.2	25.26	27.12	26.57	28.05	28.65	30.12	30.25	25.87
0 C	±0.325	±0.459	±0.246	±0.218	±0.335	±0.275	±0.251	±0.42	±0.254	±0.235	±0.347	±0.578
pH	7.33	8.68	7.82	10.02	7.72	7.76	7.51	7.62	8.05	7.81	7.64	7.08
	±0.185	±0.426	±0.565	±0.276	±0.076	±0.325	±0.427	±0.281	±0.068	±0.078	±0.216	±0.058
DO mg/L	4.48	8.48	8.81	10.16	4.64	7.71	3.04	3.31	4.81	4.65	2.94	4.22
	±0.215	±0.067	±0.229	±0.215	±0.308	±0.125	±0.232	±0.058	±0.373	±0.079	±0.305	±0.265
TA	144.08	72.74	180.3	117.55	215.03	195.6	136.4	124.2	119.7	101.23	118.75	117.86
mg/L	±1.663	±1.092	±4.532	±1.876	±1.089	±1.877	±1.642	±0.995	±0.887	±0.849	±0.559	±0.893
T H	138.72	116.82	35.64	163.26	156.42	152.3	97.02	103	93.06	83.16	93.01	110.85
mg/L	±2.125	±1.721	±1.578	±1.023	±0.675	±1.445	±1.342	±0.906	±1.097	±0.356	±0.978	±0.719

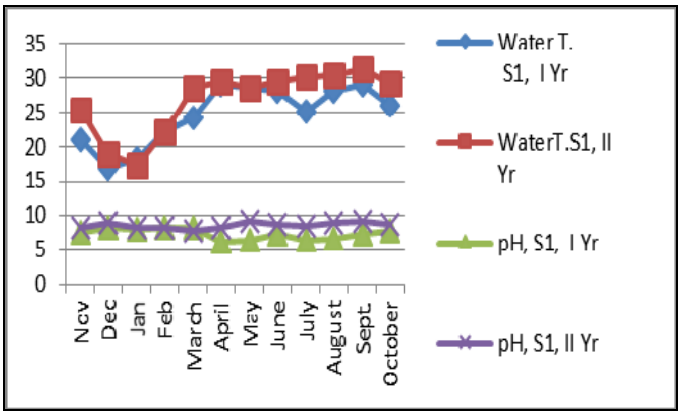


Fig 3: shows monthly fluctuation of Water temperature and pH in site1

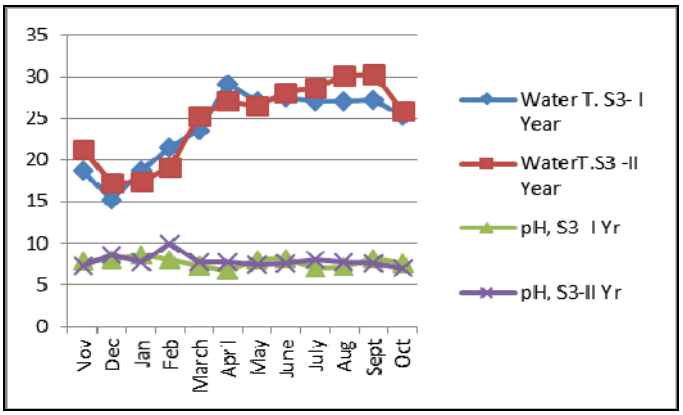


Fig 5: shows monthly fluctuation of Water temperature and pH in site 3

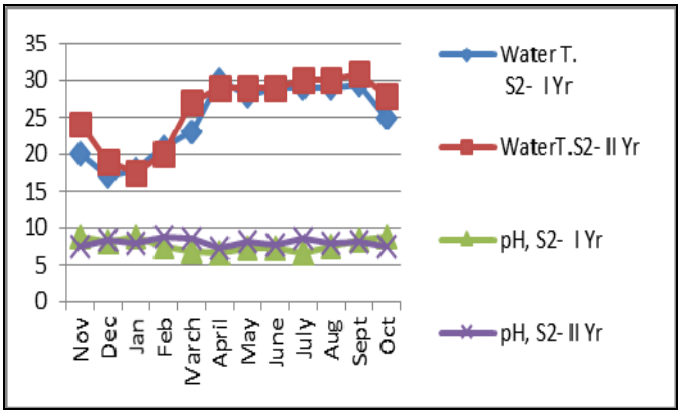


Fig 4: shows monthly fluctuation of Water temperature and pH in site 2 for two years (Nov. 2008- Oct.2010)

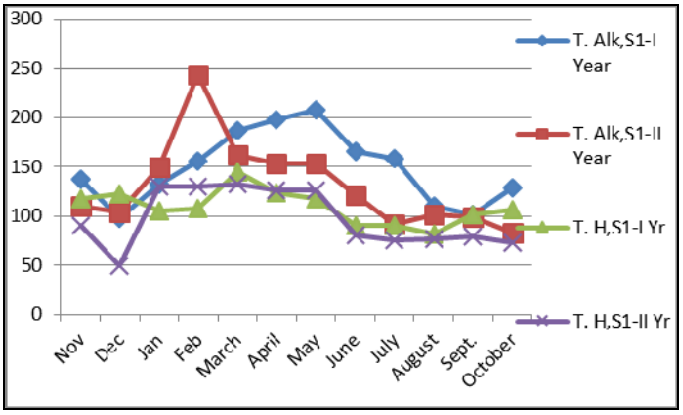


Fig 6: shows monthly fluctuation of Total alkalinity and Total hardness in site 1 for two years (Nov. 2008- Oct.2010)

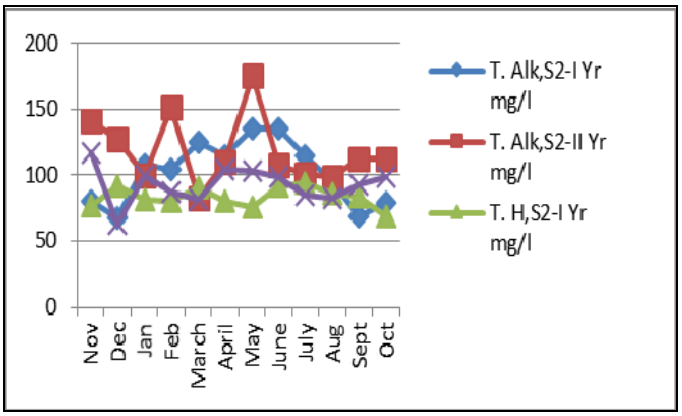


Fig 7: shows monthly fluctuation of Total alkalinity and Total hardness in Site 2 for two years (Nov.2008- Oct.2010)

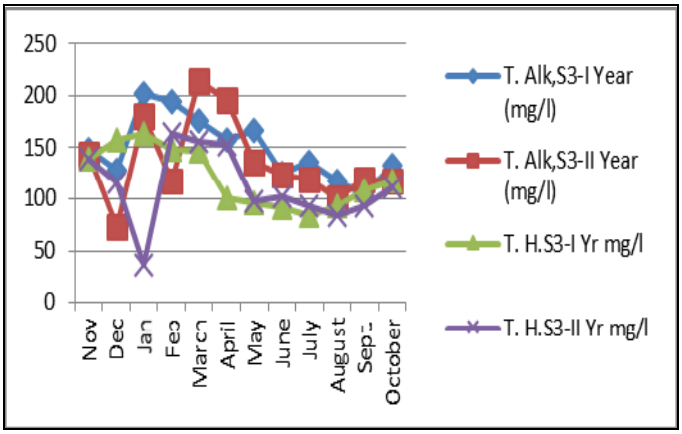


Fig 8: shows monthly fluctuation of Total alkalinity and Total hardness Site 3 for two years (Nov.2008- Oct.2010)

In site 1 and 2, water temperature was suddenly decreased in January but in site 3, WT was minimum in December and January. In site 1 and 2 both TA and TH were suddenly decreased in February last but in site 3, it was decreased in December and January (Fig. 3, 4, 7, 8). pH was generally low in all sites during outbreak of EUS(Table 1-6). Correlation coefficient was within at significant level of $P<0.01$ and $P< 0.05$. Fishes in Site 1 and 2 were heavily infected by EUS than the site 3.EUS infected fishes *Cirrhinus mrigala* (96-300 g), *Labeo bata* (45-50 g), *Labeo rohita* (30 g), *Channa* sp.(55-60 g), *Puntius* spp. (10-15 g), and *Mystus* sp. (20-25 g), *Catla catla* (340-350 g), were collected from present study sites (Fig.1 i-ix).

Hyperplasia develops between the gill lamellae. The parasites bore into the gill lamellae which perforate the blood vessels and bleeding leading to the death of fish due to suffocation. The epithelial cells locally irritated causing epithelial cellular growth and excess mucus was produced. The endomysium was infiltrated with moderate numbers of histiocytes, lymphocytes and plasma cells, with lesser granulocytes. In all cases, many fungal hyphae were associated with the ulcers, infiltrating into the myofibers and surrounding connective tissue (Fig. 2, A-H). Similar results were obtained by Pal and Pradhan [13] in *A. testudineus*, *Heteropneustes fossilis* and *Clarias batrachus* which showed ulcerative lesions on their body surface from the North Bengal. The intermediate stage lesions are represented by small (2-4 cm) dermal ulcers, associated with loss of scales, haemorrhage and oedema. In *Puntius* spp., gouramis and other mid-water fish, ulcers are particularly dark and usually circular, often a large superficial lesion on the dorsum leading to death [18].

The advanced stage lesions developed on other parts of the fishes body which expand into large necrotic open ulcers and eventual death. The Histopathology of experimentally infected *Clarias batrachus* with different bacteria isolated from EUS positive fishes which revealed vacuolation and necrosis in the liver and tubular vacuolation and necrosis in the kidney [14]. Mentioned that advanced fingerlings of Indian major carps are highly susceptible to EUS and order of susceptibility was *Catla catla*> *Labeo* spp.> *Cirrhinus mrigala*. But 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. *Cyprinus carpio* (rarely) among a total 446 affected fishes. However, only small body lesion was found in *C. carpio* which was originally known as resistant to EUS and further confirmatory test should be conducted in Cyprinus. EUS appeared prevalently in February, March-April and October-November in Nepal.

EUS pathogens also present in fresh and estuarine water bodies of the Atlantic and gulf coasts of the USA. Mass mortality of captive juvenile *Channa marulius* occurred in freshwater canals of Miami-Dade County, Florida. Histological examination revealed hyphae of *A. invadans* invading from the skin lesions deep into the musculature and internal organs [22]. Confirmation of UM associated with *Aphanomyces invadans* represents new host records in Florida for the *Archosargus probatocephalus*, *Mugil cephalus*, *Mugil curema*, *Bairdiella chrysoura*, *Pogonias cromis*, *Micropterus salmoides* and *Alosa sapidissima* [24].

The diverse group of biotic and abiotic agents, including viruses, bacteria, cutaneous ectoparasites, low pH and low dissolved oxygen concentrations, may initiate skin lesions in freshwater and estuarine fish and these non-specific lesions are subsequently colonized by *A. invadans*. Any specific environmental determinant is always associated with EUS outbreaks in freshwater or estuarine fish. It is more likely that environmental determinants will vary from outbreak to outbreak, depending on the agent initiating the non-specific skin lesions, the aquatic environment at the site and the fish populations at risk [10]. Within last 25 years EUS had been reported in more than 100 fish species at least in 24 countries in both freshwater and estuarine environments throughout south, south-eastern and western Asia, the seacoast of North America, New South Wales (NSW) Australia, Northern Territory, Queensland [9]. Little is known about the infectious diseases of native fish in Nepal. Among 16 strains of bacteria isolates, only six strains of the genus *Pseudomonas* and *Aeromonas* were the most common and opportunistic pathogens which invaded the fish secondarily. In late 2006, diseased (EUS) fish of a variety of species began to appear in the Chobe and upper Zambezi rivers in southern Africa [2].

A combination of several environmental factors (i.e. low water pH, low alkalinity, low temperature, low ambient temperature, site and sampling month) is associated with EUS outbreaks in the Zambezi River System. In June 2010, *Aphanomyces invadans* was detected in infected fishes of Barwon-Darling River using histopathology and PCR [6]. In the year 2010–11, EUS outbreaks resulted heavy fish mortality in wetland of Uttar Pradesh, India, 13 fish species were found to be infected with prevalence of about 69%. Furthermore, the disease was observed even in the month of May when the mean water temperature was 31.6 ^{0}C [15].

In the present study epizootics occurred in autumn (2008-09) and winter (2009-010) with water temperatures below 28 ^{0}C

and decreasing. Water quality variables monitored at the time of fish sampling in infected site-2 were: temperature20-23.5 ^{0}C; pH 7.5–7.7; electrical conductivity 0.14-0.35 µs/cm dissolved oxygen 5–6.9 mg/L in February (Fig.5-6). Dissolved oxygen concentrations were not below acceptable trigger values for aquatic ecosystems of heavily infected fish pond in Babia Birta, Morang. According to local fish farmer, the *Labeo bata* fingerlings were introduced from Bihar, India and suspected the transmission of EUS from there and *Cirrhinus mrigala* were highly affected. So, fascilation of EUS outbreak and transmission might have occurred due to the addition of cold water (12-17 ^{0}C) from Koshi canal during January. Among wild fish (6 spp.) *Puntius* sp., *Mystus* sp., *Heteropneustes* sp., *Channa* sp., *Amphipnous cuchia and Anabas testudineus* were found to be infected. In cultured species (12 spp.) *Cirrhinus mrigala*, *Labeo bata*, *Labeo rohita*, *Catla catla*, *Hypophthalmichthys molitrix and Clarias batrachus* were infected. *Labeo bata* and *Cirrhinus mrigala* were infected even after April when the temperature was 30-31 ^{0}C.

In the present study, the occurrence of the outbreak in all sites coincided with a sudden drop of alkalinity, temperature, pH, acidity and hardness levels of the water (Fig. 3-8). Low water temperatures (16 ^{0}C) and rapid decreases in temperature are immune suppressive and induce changes to the epidermis, including loss of mucus that predispose fish to outbreaks of EUS [5, 17]. Low pH, low dissolved oxygen, decreasing alkalinity, hardness of water will be affected in outbreaks of EUS [19]. Outbreak of epizootic ulcerative syndrome (EUS) were observed at the periphery of Baidya fish farm due to sudden fall in temperature (more than 5 ^{0}C) during winter months when polluted cold water from Koshi canal was added. The affected fishes developed several lesions around the body and fin rot [26].

Dissolved oxygen levels in the study sites were significantly lower and potentially harmful levels for fish during the outbreak than those in the period after the outbreak exerting stress on fish and weakening them. The disease outbreak was accompanied by increased BOD levels in the water which indicate organic pollution. Current findings indicate that normal skin defenses must be compromised in some way before *Aphanomyces invadans* can attach to the skin and invade underlying tissues. EUS outbreaks are usually seasonally recurrent and influenced by seasonal changes of water quality variables, play a role in lesion induction. It was reported that maximum EUS prevalence in estuarine fish populations with seasonal aggregations of fish stressed by low or rapidly changing water temperatures and rapid or prolonged depressions of salinity [18].

Aeromonads bacteria and Aphanomyces fungi have been frequently isolated from lesions of EUS-affected fishes [11, 18]. Recent studies indicate that a specific fungal pathogen, *A. invadans*, is the necessary cause of the disease [10]. Histopathological study of EUS-affected fish species in study sites revealed the presence of massive numbers of highly invasive, broad nonseptate fungi. *A. invadans* invasion in the examined fish was associated with inflammatory changes, necrosis of myofibrils and proliferative granulomatous response (Fig. 2 A-H).

4. Conclusion

The outbreaks of EUS are stress related. Whenever one or more stress factor increased, fish immune systems could not guard against the pathological agents then outbreak of the disease could be obtained. The interaction between rainfalls, deterioration of water quality especially the depletion of dissolved oxygen levels can provide stressful conditions inducing EUS lesions in susceptible fish populations. It is unlikely that any specific environmental factor and the mere presence of the pathological agents are always associated with all EUS outbreaks, if all other factors are optimal. Improving water quality management is recommended to reduce the impact of disease outbreaks. The less susceptible species cultured may reduce the impact of EUS and maintain the species diversity.

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