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Research Article

Epidemiology of Epizootic Ulcerative Syndrome in the Zambezi River System. A case study for Zambia

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Abstract. Objective: An epidemiological investigation on the fish disease Epizootic Ulcerative Syndrome (EUS) was conducted from January to December 2011 in the Zambezi River System (ZRS) of Sesheke District in the Western Province of Zambia. The study aimed at determining: factors associated with outbreaks of EUS, infection rate and distribution of the disease. EUS is a newly confirmed disease in Southern Africa caused by a fungal pathogen, *Aphanomyces invadans*. Material and Methods: Active surveillance was conducted where a total of 4,800 fish were inspected in February, June and October for gross EUS-like lesions and disease confirmed using histopathology. Environmental cues were also assessed monthly for one year to determine their association with disease outbreaks. A questionaire was adminstered to assess spread of EUS while Geographic Information System helped map disease distribution. Results of the study implicate several predisposing environmental factors; heavy rains preceded outbreaks resulting in excess flooding which caused water levels to rise 2m higher than normal; predominantly gleysol and arenosol soils of the ZRS resulted in low water pH (4.53 to 6.5). Other factors significantly ($p<0.05$) associated with EUS outbreaks were: low total alkalinity (45.13±SE 0.0418), water temperature (20.94±SE 0.2173), ambient temperature (25.85±SE 0.3058), month (June) and site (lagoons) of sampling. Infection rate was 3% (144) of the 4,800 fishes sampled. Of these, 58 (40.2%) had mycotic granulomas after histopathological analysis, representing 1.2% of the total sample. Eighty six (86) of the 144 fishes were diagnosed with healing wounds representing 1.8 % of the total fish sampled. Some of them, 4,656 (97%) more exactly, had no gross lesions. Conclusion: There is indication that EUS has affected fish in ZRS from Kazungula to Chavuma Districts of Zambia with sub optimal environmental factors being associated with disease outbreaks.

Key Words: environmental cues, fish, fungus, histopathology, infection rate

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Introduction

Many communities living along the rivers and lakes in Southern Africa and Zambia, in particular, have enjoyed fish without any worries of fish diseases until when the first outbreaks of Epizootic Ulcerative Syndrom (EUS) in Africa (Zambia, Botswana and Namibia) were reported in 2006. Diseased fish of a variety of species began to appear in the Zambezi Fishery resulting in a massive fish kill. The biodiversity of the fishery was being threatened and posing food insecurity to over 2000 villages and some 700,000 people with Zambia being most affected (FAO 2009). Formalinised fish samples were collected from Sesheke District of Zambia and sent to the University of Zambia on suspicion of chemical poisoning or anthrax contamination (Samui *et al* 2007). Similar outbreaks were reported from Botswana and Namibia and investigations showed that the levels of pesticides and heavy metals in the tissues of the fish were very low thus, discounting pollution as an underlying cause of disease. In May 2007, collaborative works between the University of Zambia surveillance team and the EUS Reference Laboratory in Thailland confirmed the occurrence of *Aphanomyces invadans* (a fungal pathogen which causes EUS) in fish of the Zambezi River System (Samui *et al* 2007).

Further surveillance works conducted in 2007 by the same surveillance team (University of Zambia), estimated EUS infection rate per catch to be as high as 50 percent in newly infected areas of Zambezi and Chavuma Districts (FAO 2009), whereas by 2008, an infection rate of only 5 percent per catch was observed in Sesheke District where the disease was first noticed in Zambia (FAO 2009). This is an important observation as it implies decreasing disease intensity with time (Roberts *et al* 1994; Lilley & Roberts 1997; Lilley *et al* 1998; Baldock *et al* 2005). However, not much epidemiological research had been carried out since then to adequately elucidate factors associated with EUS outbreaks in the Zambezi River System hence necessitating the current study with the major objective to investigate and better understand the epidemiology of the disease in the System in Zambia and specific objectives to:

i. determine the environmental and biological risk factors associated with EUS outbreaks in the Zambezi River system of Zambia;
ii. determine the infection rate and severity of EUS in fish of the Zambezi River System;
iii. determine the spatial distribution of EUS in the Zambezi River System of Zambia.

Materials and methods

Environmental risk factors

Hydrological sampling

Water sampling was done in the morning (07:00 - 08:30) of the 15th day of every month from each of the sampling sites (Fig. 2) of the study area (Fig. 1). Water samples were collected using a plastic Van dorn Sampler which was lowered to desired depths of 0 m, 1 m, 2 m, 3 m, 4 m and 5 m and then closed off by activating a drop weight (messenger) which slides down the rope. To minimise sampling errors replicate samples were collected and measured for all parameters (water temperature, oxygen, pH, ammonia, nitrite, nitrate, salinity and total alkalinity). Biological activity in the collected samples was reduced by storing samples at low temperatures in an iced cool box and kept in the dark as much as possible from the time of collection until analysis. Field analyses were done within a few hours.

Figure 1. Location of study area in Zambia and Southern Africa (map adapted from www.africamap.com)

Water temperature

An electronic digital thermometer (Bravo sharp) was used to measure water temperature for every level of the water column sampled with the Van dorn sampler.

Dissolved oxygen

A portable oxygen meter (HI 8543) was inserted in the respective samples and readings recorded respectively, from water surface to 5m.

pH

A portable pH meter (PH-8414) with a temperature sensor was used in the analyses. Before pH measurements, the meter was calibrated using buffer 4.0 as the pH unit within the range for the target sample waters. The water for testing was sampled at varying depth i.e. 0 m, 1 m, 2 m, 3 m, 4 m and 5 m using the Van dorn sampler.

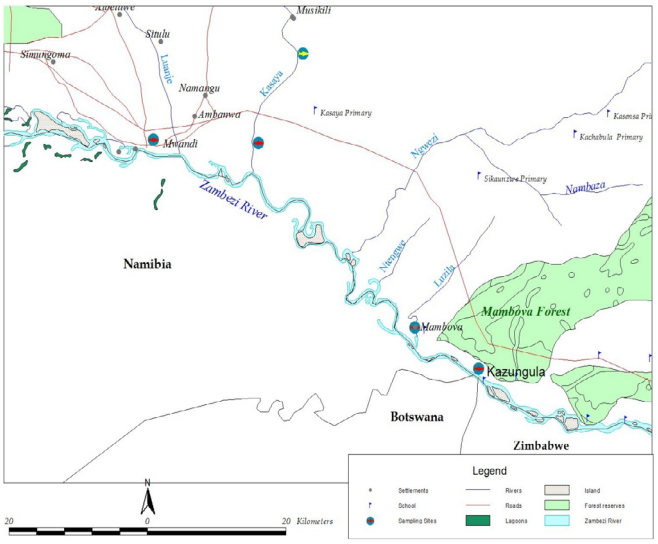

Figure 2. Sampling sites

Salinity

As water was sampled for all other parameters, a drop of water sample from each level (0m, 1m, 2m, 3m, 4m and 5m) was put on a hand refractometer (MT-110 ATC) and the salinity reading was instantly recorded in ppt.

Total alkalinity

After sampling water with a Van dorn sampler from respective water levels (0 m, 1 m, 2 m, 3 m, 4 m and 5 m), two 250 mL glass bottles were filled to the brim without aeration. The sample bottles were then sealed and kept ice cold in a cooler box until analysis time. Analyses of samples were carried out within a few hours of collection.

Alkalinity was determined by titrating water samples with a standard solution of 0.02 N sulphuric acid. A 100 mL of sample water was gently poured into a 250 mL beaker and sample pH recorded using a pH meter while shaking gently. Duplicate samples were analysed.

Titration using a standard solution then followed and the titrant volume was recorded while shaking gently up to pH 4.5 which is a bicarbonate end point. Total alkalinity was then calculated as below following the protocol by Lind (1985).

Total alkalinity as mg $CaCO_3$ per litre = B x N x 50,000 mL sample; where: B = mL total titration from start to pH 4.5; N = Normality of acid.

When titrating a 100 mL sample with 0.02 N H_2SO_4, the equation is Total alkalinity as mg Ca CO_3 per litre = B x 10

Ammonia nitrogen

Using a Van dorn sampler, water samples were collected from respective water levels (0 m, 1 m, 2 m, 3 m, 4 m and 5 m). An ion electrode type meter (oaktron- ion meter-TiN 9001) specifically designed to measure ammonia was used for instant field readings.

Measuring nitrogen as nitrate using Nitrate Nitrogen Tablet Kit
1) A nitrate tablet was added to a vial containing 5 mL of sample water. The vial was then capped and shaken until the tablet disintegrated.
2) Another nitrate tablet was again added as tablet no. 2, the vial capped and shaken until the tablet disintegrated.
3) The sample was then mixed and left for 5 minutes.
4) First a Nitrate-Nitrogen Ocat-Slide Bar and then the vial were inserted into an Octa-Slide Viewer (Color Comparitor) and the sample color matched to a color standard and recorded as ppm Nitrate-Nitrogen.
For nitrite nitrogen step 2 was repeated (according to Burres 2010).

Quality assurance and quality control
The quality-control was designed to incorporate replicate samples for accuracy purposes. Equipment-blank samples of deionized water certified to be free of organic and inorganic compounds were passed through all sampling equipment during the sampling period to verify initial cleanliness. A field-blank sample was used to demonstrate that the equipment used in sampling was adequately cleaned to remove contamination introduced by previous use, ensure that sample collection and processing had not resulted in contamination and that sample handling, transportation, and laboratory analysis did not introduce contamination. The objective of collecting replicate samples was to estimate the precision of concentration values from sample processing and analysis.

Meteorological data
Secondary data on rainfall was obtained from the meteorological station in Sesheke District within the Zambezi River System compiled for a 10 year period starting 200 L through 2011 (Fig. 3) while ambient temperature data was collected during the one year span of the current research.

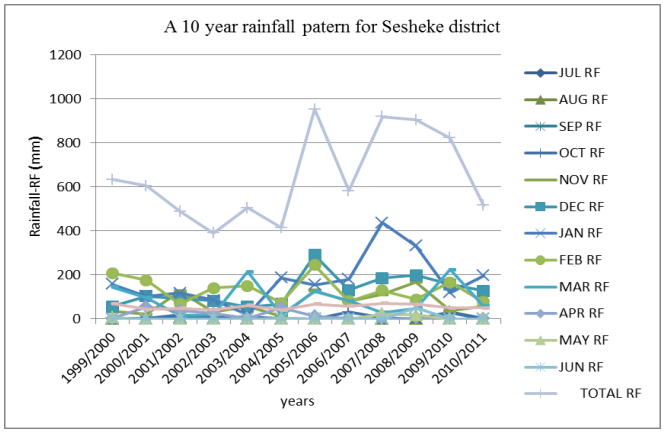

Figure 3. Rainfall trend for Sesheke District for ten consecutive years

Biological risk factors

Sampling Fish
At each study site at least 320 fish specimens were sampled using a 12mm meshed seine net. Water quality parameters were measured prior to fish sampling. The seined fishes were then put on a flat but smooth wooden surface and separated by species using a fish guide. Information gathered with reference to species, size, fork length and sex of fish was entered in an information sheet and the fish was then checked for lesions . Any fish showing lesions or ulcers resembling those associated with EUS was considered suspect and was categorised under level 1 examination which involved gross observations as detailed in the OIE manual of diagnostic tests for aquatic animals (OIE, 2006). In some cases, inspections were done on fish catches directly from fishermen and any apparently infected fish were purchased and prepared for examination.

Table 1. Component matrix for 13 environmental cues

	Component		
	1	2	3
Site	-0.099	-0.093	0.833
Ambient temp	0.113	0.944	0.105
Water flow	-0.467	0.334	0.452
Water level	-0.937	-0.236	-0.091
Temperature	-0.483	0.839	0.012
Oxygen	0.169	-0.912	-0.086
pH	0.111	-0.507	0.614
Ammonia	0.895	0.189	0.085
Nitrite	-0.174	0.682	-0.145
Nitrate	0.023	-0.273	-0.425
Salinity	0.89	0.232	-0.026
Total alkalinity	0.903	0.369	0.029
Month	0.909	-0.162	0.059

All the fish showing lesions were then prepared for level 2 examination (histopathology) as follows;
1. Taking a photo of the fish
2. Recording species, fork length, sex and any other observation of relevance
3. Dissecting the lesion and puting it in a labeled bottle containing 10% buffered formalin. The labels recorded at least the date, location, fish ID and species. On the other hand, fish not showing lesions were only measured for length by species. Where less than 320 fish were collected with the first seining, the same net was repeatedly used around the sampling site to bring the sample to at least 320.

Figure 4. EUS lesion on *Brycinus lateralis*

Figure 5. EUS infected *Sargochromis giardii*

Figure 6. Healing wound on *S. robustus*

After processing the 320 fish, any slow moving and sick-looking fish along the edge of the water body was also collected using a scoop net. After sampling in one site, the seine net and scoop net were dried under the sun for at least 2 hours before using them in a different sampling site. The Collected samples were transported to the University of Zambia for further processing. The sampling procedure was as recommended by Corsin (2007). In addition to the active surveillanve above, questionnaires (participatory epidemiology) were also used to interview a total of 350 fishers regarding EUS and how to recognize it; their opinion on EUS outbreaks and its distribution; the need to report suspect EUS cases as this was extremely important for the management of the disease and the need to collect fish showing lesions and submit them to the local fisheries officer. Fisheries officers and fishers were trained on how to preserve the EUS infected fish samples using the procedure outlined above.

Statistical analysis

Principal Component Analysis was used to reduce 13 environmental parameters initially percieved as significant to EUS occurrence in the Zambezi River System to 6, while Logistic Regression Analysis was used to test the significance of these final parameters at $p<0.05$ (Table 2) and finally, GIS was used in mapping the distribution of disease after establishing cordinates for the sampling stations using the GPS (NAVI 1300).

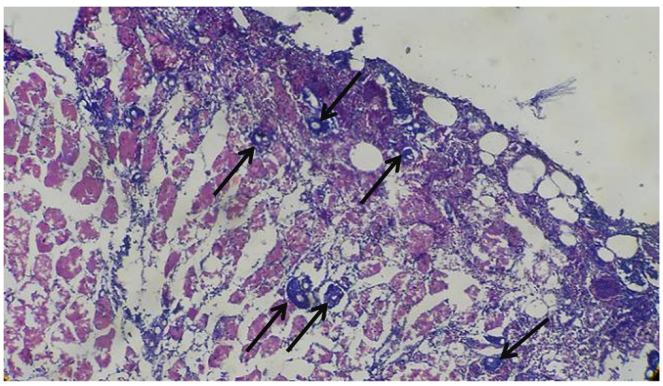

Figure 7. Histopathology of EUS-infected *Brycinus lateralis* showing typical mycotic granulomas surrounding invasive fungal hyphae (black arrow) penetrating into muscle layer (H&E stain)

Results and discussion

Environmental risk factors

There is indication that outbreaks were preceded by heavy rains (Fig. 3) resulting in excess flooding in the Zambezi plains causing water levels to rise 2 m higher than normal. Water pH was found to be significant (5.4 ± SE 0.0199) which is ideal for the EUS causing agent to sporulate. Other factors significantly ($p<0.05$) associated with the EUS outbreaks were, low total alkalinity (45.13± SE 0.0418), water temperature (20.94 ±SE 0.2173), ambient temperature (25.85± SE 0.3058), month of June (cold season) and site of sampling (lagoons, oxbow lakes and tributaries being significant).

Infection rate and severity of disease

Gross examination estimated the infection rate at 3% (144) of the 4,800 fish sampled of which 58 (40.2%) were found to have mycotic granulomas after histopathological analysis representing 1.2% of the total fish sampled during the research. The remaining 86 of the 144 fishes were diagnosed with healing wounds representing 1.8 % of the total fish samples recorded. Some of them, 4,656 (97%) more exactly, showed no evidence of gross lesions.

Disease severity in fish was generally mild and no mortalities were observed during the research. Out of some 15 species sampled, *Brycinus lateralis*, *Serranochromis robustus* and *Sargochromis giardii* seemed to be the most susceptible to EUS (Figs. 4-6).

Histopathology

Histopathological studies of lesions on ulcerated fish showed diffuse infiltration of broad, non-septate fungal hyphae and a large number of inflammatory cells. Muscle tissue necrosis and hyperaemia were also observed in the lesions. All fish with lesions examined showed similar pathology (Fig. 7).

EUS distribution

Participatory epidemiology indicates that all the districts within the Zambezi River System in North Western, Western and a part of Sothern Provinces, i.e. Chavuma, Zambezi, Kabompo, Lukulu, Mongu, Kalabo, Senanga, Sesheke and Kazungula, were either directly or indirectly affected. The affected areas include all major

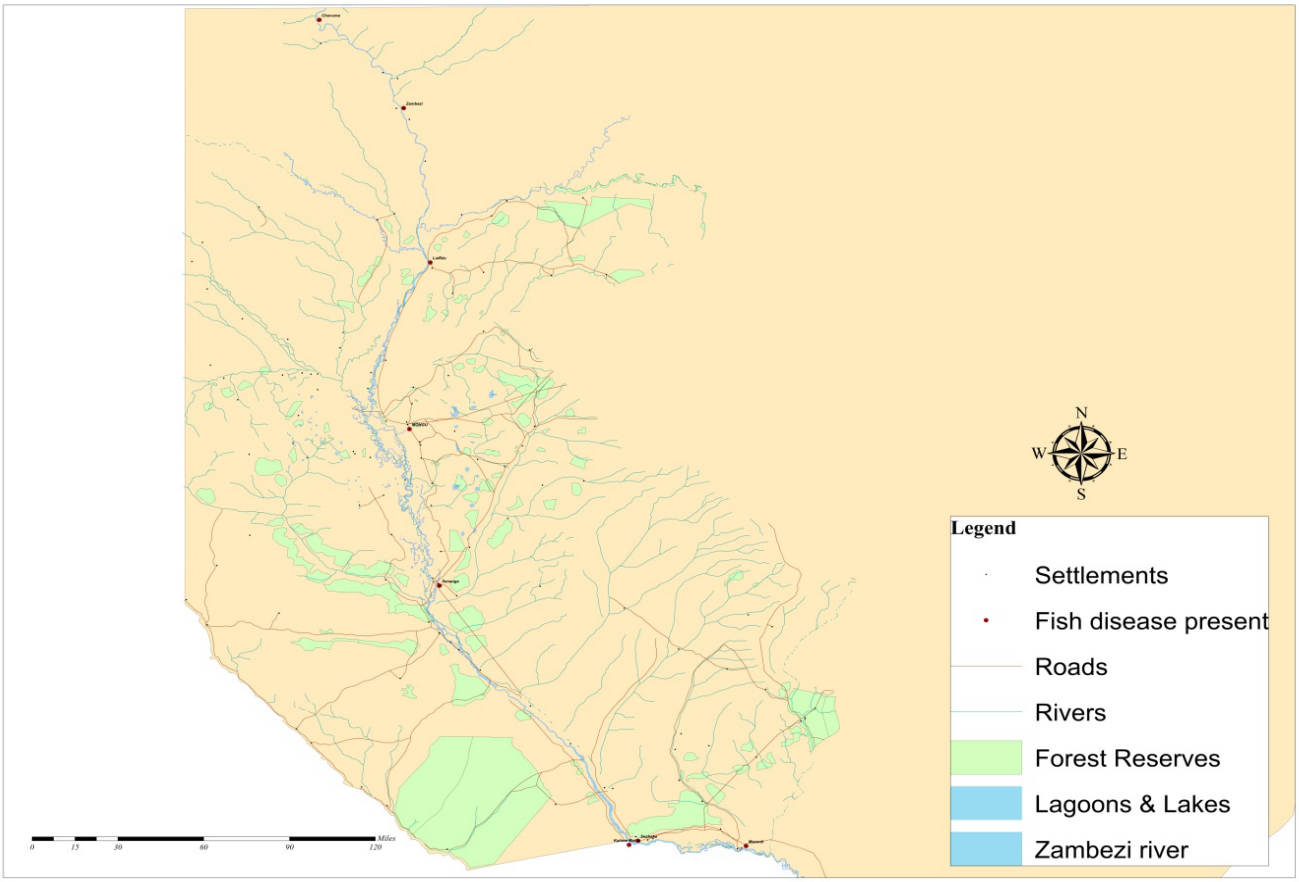

Figure 8. Disease distribution in the Zambezi River System of Zambia

water bodies in the plains as well as in the uplands. Regarding the sampling sites in Sesheke, field observations indicate that disease is more in the lagoons, oxbow lakes and tributaries than in the backwaters and Main River and that diseased fish in the main river were a spill over from the lagoons, oxbow lakes and tributaries where diseased fish were recorded in the month of June. Disease distribution therefore stretches from Kazungula District of Southern province, through Western Province to Chavuma District of North Western province of Zambia covering the entire Zambezi River System in Zambia (Fig. 8).

Participatory epidemiology

Three hundred and fifty (350) fishers were interviewed across the 8 Districts of Kazungula, Sesheke, Senanga, Mongu, Lukulu, kalabo, Zambezi and Chavuma during the research and made the following observations and opinions about risk factors associated with EUS outbreaks in the Zambezi River System:

1. EUS outbreaks have occurred every year since 2006 and affect most of the fish species;
2. Initially the severity of disease was characterised by heavy mortalities, but this has reduced to mild red spots with no deaths recorded;
3. Two hundred (200) fishers reported that outbreaks occur every year in their local fishing areas as the temperature begins to drop and that all fish are affected with the small sizes being infected most;
4. EUS often occurs in lagoons, oxbow lakes and tributaries not very close to the main river system;
5. Some fishers were of the opinion that fish-eating birds and reptiles such as crocodiles might be transmiting the disease from one place to another by preying on easy to catch EUS-affected fish and dropping uneaten portions in unaffected water bodies;
6. Most fishers interviewed believed that floods play a vital role in spreading EUS throughout the Zambezi River System;
7. Some observers thought that moderately affected fish might transmit the disease by entering an unaffected water body;
8. EUS was associated with low temperature and reportedly occurred after periods of heavy rain.

Statistical analysis

The risk factors included in the final model are presented as coefficients and odds ratios with their 95% confidence intervals:

$$\text{Logit } \{P(x_1, x_2.....x_6)\} = \alpha + (\beta x_1) + (\beta x_2) + (\beta x_3) + (\beta x_4) + (\beta x_5) + (\beta x_6)$$

Where: x is the hypothesised risk factor; X_1 = Site of sampling; X_2 = Month of sampling; X_3 = Ambient Temperature; X_4 = Water Temperature; X_5 = Water pH; X_6 = Total alkalinity; α = Intercept; β = Coefficient; P = Probability of the disease to have really occurred where it is detected by a positive histopathological result.

The study results implicate a combinations of 6 environmental cues (Table 2) as being risk factors associated with EUS outbreaks in the Zambezi River System. Adverse meteorological and hydrological conditions negatively affected the system and

subsequently compromised fish health. Low water pH caused scale removal and burns on the skin of fish thereby providing portals of entry for opportunistic disease agents. This coupled with other factors in Table 2 then favoured *Aphanomyces invadans* which caused skin ulceration and bloody patches on the body of fish leading to EUS outbreaks.

Table 2. Means for environmental cues and measures of association with disease

Environmental cue	No. of sampling	Mean	Standard error	Sig[a]	Measure of association with disease
Month	321	6.028	± 0.1861	0.003	0.922
Site	321	2.996	± 0.0790	0.049	0.604
Air Temperature	321	25.85	± 0.3058	0.04	0.906
Water Temperature	321	20.94	± 0.2173	0.02	0.889
Water pH	321	5.4	± 0.0199	0.016	0.782
Total Alkalinity	321	45.13	± 0.0418	0.007	0.743

[a]$p<0.05$

Secondary data on rainfall obtained during the current research from Sesheke metrological station shows 952.6 mm of rainfall in the Zambezi River System in Zambia during 2005/2006 rainy season when EUS was first reported. This figure is above the 500 mm average of the area and the highest in the previous 10 years (Fig. 3). This undoubtedly impacted on other hydrological variables such as the ones in Table 2 above showing sub-optimal values below what is required for fish health. Consequently the fouled aquatic environment stressed the fish and compromised their immunity. The declined environmental conditions however favored the sporulation of the opportunistic pathogen (*Aphanomyces invadans*) which is ubiquitous in most freshwater bodies. The pathogen then easily infected already stressed fish leading to EUS outbreaks.

In the present study, detailed analysis of monthly fluctuations of ambient temperature in the study area revealed that the fluctuations during the outbreak period were significantly lower (25.85° C) than those in the period before and after disease outbreaks and therefore associated with EUS outbreaks. This is in agreement with the report in Philippines and Bangladesh, where EUS outbreaks also occurred at times of significant montly fluctuations in ambient temperature (Phillips 1994). Such ambient temperature fluctuations would stress the resident fish populations in the area, leading to EUS outbreak and spread of the diseases. The current study observed an average water temperature of 20.94° C and therefore falling within the range recorded in previous works. For example, according to Chinabut *et al* (1995), in environmental conditions of the subtropical region, where the average temperature is about 26° C, the temperature range for both fungal growth and sporulation of *Aphanomyces invadans* results in high risk of EUS outbreak and spread. Willoughby (1994) and Willoughby & Roberts (1994) also reported a wide range of water temperatures, 10-37° C for the growth of the fungus. Heavy rainfall in an area can bring the risks of water quality deterioration, directly through runoff and indirectly by introducing infectious agents into the waterways. In the present study, analysis of the secondary data on rainfall pattern of the area revealed that EUS outbreaks occurred during the dry season, starting immediately after the rainy season. The outbreak stopped at the onset of the following rainy season.

Seasonal occurrence of EUS was recorded in the present epidemiological study with occurances being recorded in the cold season. This agrees with other findings such as in Southeast Asian countries (Bondad-Reantaso *et al* 1992; Phillips 1994) that confirmed that seasonality is one of the key features of EUS outbreaks. In the current research environmental analyses of water quality parameters in relation to EUS occurrence showed that EUS outbreaks were associated with decreasing alkalinity and water temperature and this is also consistent with other findings (Bondad-Reantaso *et al* 1992; Phillips 1994; Samui *et al* 2007). Also in Bangladesh, Sanaullah *et al* (2001) found that a rapid decrease in water temperature, and alkalinity with respect to hardness were significant stressors predisposing fish to EUS. The current research discovered that the EUS outbreaks were significant in lagoons, oxbowlakes and tributary than other sampling sites, this is owing to the evidence of concentrated elements during the dry season when water levels are significantly low. It was found that water pH was lower than in the main stream where it was close to neutral and therefore implicated as one of the predisposing factors to the disease outbreak. This finding is similar to reports in Australia and the Philippines where outbreaks of EUS, were associated with acidic water during periods of heavy rainfall (Sammut *et al* 1996). Secondary infectious agents such as *Saprolegnia, Aeromonas hydrophila* and *Aphanomyces invadans*, which are normally opportunistic pathogens of EUS, were previously isolated microbiologically from some fish samples (Samui *et al* 2007).

The outbreaks of EUS are stress related. It is unlikely that the mere presence of the pathological agents would lead to the disease if all other factors are optimal. Whenever one or more stress factor increased in magnitude such that fish immune systems could not guard against the pathological agents, then an outbreak of the disease could be observed. The present study indicates that interaction between ambient temperature, water temperature, water pH, low alkalinity levels, sampling site and month of sampling (seasonality) provided stressful conditions for fish, thereby inducing EUS lesions in susceptible fish populations thereby leading to EUS outbreaks. It is unlikely that any specific environmental factor is always associated with all EUS outbreaks. Therefore, further studies under laboratory and held conditions are needed to investigate further on more environmental risk factors that lead to EUS and to discover their exact role in the EUS outbreaks.

Furthermore, the absence of adequate data on the relationship between EUS and the environment calls for continuous and region-wide monitoring programme of selected environmental parameters to help in elucidating these variables (Rahman *et al* 2010; Aftabuddin & Akter 2011; Crottini *et al* 2011; Sankar *et al* 2011).

It is envisaged that results from the current research would have a wide application in formulating intervention strategies which would help mitigate the disease impact in newly affected water bodies and minimse the possible spread of EUS to other aquatic ecosystems which are not yet affected.

The study demonstrated that several predisposing environmental factors are associated with EUS outbreaks among fish in the Zambezi River System. Although control of EUS in the wild is difficult, successful prophylactic treatments can generally be administered by the Department of Fisheries together with local communities involving addition of agricultural lime mainly in lagoons and oxbow lakes before they get cut off from the main river system. This would be a relatively simple and inexpensive way of enhancing water quality thereby reinforcing the need to overcome the environmentally degrading conditions which may predispose fish to EUS infections.

Biosecurity of the Zambezi River System must be given attention by instituting appropriate management and policy measures such as restriction of trans-boundary fish stock transfers (Musuka & Musonda 2012). More often than not fish stocks earmarked for aquaculture purposes are collected from the wild and therefore observance of the restriction to fish translocation must be upheld. Epidemiologists must be empowered to formulate and reinforce legislatory measures to this effect. The research results provide a multifaceted benefit to various stake holders such as the Department of Fisheries who should device plans to reserve species diversity of the ZRS following EUS outbreaks. Bilateral agreements between Governments must focus more on preventing the disease spread to non-affected areas with similar environmental conditions as the ZRS. Rainfall higher than average 500mm must serve as a warning alarm to the recurrence of EUS outbreaks in the ZRS.

Conclusions

In conclusion, unlike other EUS outbreaks in other parts of the world, the current study has demonstrated that a combination of several environmental factors (low water pH, low alkalinity, low temperature, low ambient temperature, site and month of sampling) is associated with EUS outbreaks in the Zambezi River System. The fouled aquatic environment created by sub-lethal physical and chemical conditions caused trauma to the fish and subsequently compromised immunity allowing the opportunistic fungal pathogen to infect the fishes. This knowledge is important for stakeholders who are trying to manage fisheries and or promote aquaculture as they have an idea of predisposing environmental conditions for EUS outbreaks. More studies are however required to determine which other fishery areas of Zambia have similar environmental conditions as the ZRS to help minimize the spread of the disease. Humans should not eat fish suffering from EUS. While there is little risk of the disease infecting humans, previous works (Samui *et al* 2007) indicate that fish with an advanced EUS case carry bacteria in the skin lesions that can make humans ill.

Acknowledgements

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