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Impacts of Extreme Cold Water Conditions and Some Bacterial Infections on Earthen-Pond Cultured Nile Tilapia, *Oreochromis niloticus.*

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ABSTRACT

Tilapia aquaculture is vulnerable to adverse impacts of unfavorable environmental conditions and microbial agents. Over-wintering is a serious economic challenge in tilapia farming. Exposure to extreme cold water temperature has long been considered a significant constraint to tilapia fish survival. Unprecedented huge mortalities approached 98 % have been recorded only in earthen-pond cultured tilapia, *Oreochromis niloticus* within Barsiq farm, northern Egypt during the period from December 2013 to February 2014. Other co-cultured fishes weren't surrendered. Succumbed fishes showed distinctive skin darkness and few haemorrhagic patches on the external body surface. The synergistic effects of environmental deterioration and opportunistic pathogens have been accused for these substantial losses. Water temperature approached detrimental levels 5.2 °C. Mortalities were severe in young-aged tilapia 95 %. Losses approached high rats 98 % particularly in shallow water ponds 55 cm in depth compared to 30 % in other ponds (more than 100 cm). The values recorded for dissolved oxygen, NH_3, NO_2 and NO_3 were fare from the optimum recommended levels; 3.5 mg/L, 1.03 mg/L, 0.75 mg/L and 10.1 mg/L respectively. The magnitude of stress formula exaggerated by existence of unfavorable levels of some heavy metals. *Vibrio anguillarum* and *Aeromonas hydrophila* were isolated from succumbed fishes.

Keywords: *Oreochromis niloticus*, Extreme cold water conditions, Bacterial infections, High mortalities.

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INTRODUCTION

Fisheries and aquaculture make critical contributions to the world's wellbeing and prosperity. Egypt's fishery sector is a fundamental element of the national culture and economy [1]. The country has established the leading aquaculture industry in Africa. Virtually fish farming has been practiced in Egypt on the banks of River Nile more than 4,000 years ago. Furthermore, the earliest recorded history of fish keeping in ponds in Egypt is depicted on the walls of the Tomb of Thebaine [2, 3].

The country put fish farming sector on the center of development in recent years after aquaculture has been proposed as the ideal pathway to cover the gap between supply and demand for fish in Egypt [1, 4]. Tilapias are one of the most crucial warm water fishes used for aquaculture all over the world. In Egypt, tilapia farming, has witnessed a spectacular expansion resulting in an enormous industry that rank second globally and the first in the MENA region [5, 6].

Egyptian tilapia aquaculture started with the use of traditional extensive and semi-intensive techniques. Later, implementation of intensive rearing methods and a shift to faster growing species, monosex tilapia, led to a boom in this aquaculture industry [7].

One major dilemma to the global expansion of tilapia farming is their maximized sensitivity to low ambient temperature. Exposure to extreme cold conditions leads to tilapia mass mortality rendering over-wintering a serious economic challenge in tilapia farming [8, 9, 10, 11]. The aquatic environment plays an important role in the pathogenesis of fish diseases. The impacts of unfavorable environmental conditions on diseases noticed in fish stocks have been well documented. The synergistic effects of environmental deterioration and opportunistic pathogens have been accused for substantial fish losses noticed in many farms [1, 4, 12].

The present study was conducted to investigate the environmental and accompanied bacterial causes provoked tilapia mass mortalities noticed in Barsiq farm located at Behiera province northern Egypt during the extreme cold water period extended from the end of December 2013 until February 2014.

MATERIAL AND METHODS

Area of study and case history

Barsiq farm is located south to Lake Edco Behiera province, Egypt. It has an approximate area of 2,000 acres. It includes tilapia hatchery ponds on the basin area of 10 acres, ponds for wintering tilapia on an area of 10 acres, ponds for brooding on an area of 88 acres and production ponds about 1144 acres. *Oreochromis niloticus , Mugil cephalus and Cyprinus carpio* are the main cultured fish species [7].

Unprecedented high mortalities noticed only in *Oreochromis niloticus* but weren't recorded in other ponds cultured with *Mugil cephalus* and *Cyprinus carpio*. Losses erupted at the end of December 2013, when water temperature was below 8°C and persisted until February 2014, when water temperature increased up to 13°C. Mortalities were severe (95 %) in young-aged fishes (maximum 40 g) compared to (65- 70 %) in adults (more than 150 g). The depth (water column) of ponds ranged between 55 cm to 1.5 m. One hundred freshly dead and /or moribund fish specimens from affected ponds were transferred to the laboratory of Hydrobiology Department, National Research Centre, Egypt for bacteriological investigation with the minimum time of delay. The body weights of fish samples ranged between 35-220 g.

Water quality examination

Water samples were obtained from different locations within each pond during mortalities. Three samples were collected in sterile plastic bottles for physical, chemical and heavy metals evaluations. Temperature, dissolved Oxygen (DO) and pH were measured on spot in fish ponds while un-ionized ammonia (NH_3), nitrites, nitrates and heavy metals (Iron, Copper, Zinc, Cobalt, Cadmium, Chromium, Nickel, Manganese and Lead) were measured in laboratory according to methods adopted from [13].

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Bacteriological examination

Sampling and processing

Samples were obtained from gills, liver, kidney and spleen under complete aseptic condition for bacteriological examination. Loopfuls from the above mentioned tissues were cultured onto tryptic soy agar, thiosulphate citrate bile salt sucrose agar (TCBS), pseudomonas agar base medium supplemented with CFC (cetrimide, fusidin and cephaloridine supplement, MacConkey Agar and aeromonas agar base supplemented with ampicillin. The inoculated plates were incubated at 25 °C for 18-48 hours. Representative numbers of the different colonial types detected on the media were collected from plates and streaked on TSA for purity and identification.

Identification of isolates

Identification of pure bacterial isolates was performed by studying their morphological and biochemical characteristics using traditional as well as API 20 E kit (Bio Merieux) following the criteria described in [14] **.**

Histopathological examination

Tissue specimens for histopathological techniques were taken from gills, liver, spleen and kidney of infected fishes. Samples were trimmed and fixed in 10 % phosphate buffered formalin for 24 hours, then dehydrated by a series of upgraded ethanol solution, embedded in paraffin, and sectioned at 5 µm thick. Tissue sections were routinely processed and stained with Hematoxylin and Eosin (H & E) and examined by light microscopy according to [15]**.**

RESULTS

Water quality measures

Mortalities were severe especially in shallow ponds (Fig.1). Losses approached high rats 98 % particularly in earthen-ponds with water column of 55 cm compared to 30 % in other ponds (more than 100 cm). Unfavorable average values of water quality parameters were recorded in the investigated fish ponds (Table 1). Water temperature was extremely fare from the optimum levels recommended for cultured tilapia, 5.2°C. Furthermore, dissolved oxygen levels and pH values were 3.5 mg/L and 8.18 respectively.

Table 1: Water quality measures recorded in the investigated earthen-ponds during mortalities

Item	Results (Average)	Item	Results (Average)
Temperature	5.2 °C	Cobalt	0.52 ppm
pH	8.18	Copper (Cu)	0.02 ppm
Dissolved oxygen (DO)	3.5 mg/L	Iron (Fe)	2.16 ppm
Total solids (TS)	1939.2 mg/ L	Lead (Pb)	0.48 ppm
Soluble Cations (mEqu /L)		Manganese (Mn)	0.24 ppm
Ca^{++}	3	Nickel (Ni)	0.87 ppm
Mg^{++}	21.5	Zinc (Zn)	0.37 ppm
Na^{+}	6.5	Chromium (Cr)	0.04 ppm
K^{+}	0.75	Cadmium (Cd)	0.14 ppm
Soluble Anions (mEqu /L)		Phosphorus (P)	0.168 ppm
$CO_3^{=}$	-	Ammonia (NH_3)	1.03 mg/L
HCO_3^{-}	4.2	NH_4	8.4 mg/L
Chlorine (Cl^{-})	19	Nitrite (NO_2)	0.75 mg/L
Sulphate ($SO4^{=}$)	8.55	Nitrate (NO_3)	10.1 mg/L

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Figure 1: (a) Shallow earthen-pond at Barsiq farm with too little water column. (b,c,d) High *O. niloticus* mortalities recorded at Barsiq farm Behiera province, Egypt during the extreme cold period

Results also demonstrated that nitrogenous waste products may be accused for magnifying the magnitude of stress upon fish. The values recorded for NH_3, NO_2 and NO_3 were 1.03 mg/L, 0.75 mg/L and 10.1 mg/L respectively. On the other hand, Achieved results also indicated possible involvement of some heavy metals in rendering fishes more susceptible to such mortalities. Levels of some detected metals were higher than the high reliability trigger value recommended. The recorded measures for iron, Copper, Zinc, Manganese, Cadmium, Chromium, Cobalt, Lead, Nickel, Were; 2.16 ppm, 0.02 ppm, 0.37 ppm , 0.24 ppm, 0.14 ppm, 0.04 ppm, 0.52 ppm, 0.48 ppm, and 0.87 ppm respectively in sequential table 1.

Clinical signs and P.M lesions

Skin darkness was characteristic. Some fish showed haemorrhagic patches on the external body surface, erosions and ascites. The kidneys were congested. Moreover, liver was pale and friable splenomegaly and gall bladder distention were distinctive (Fig.2)

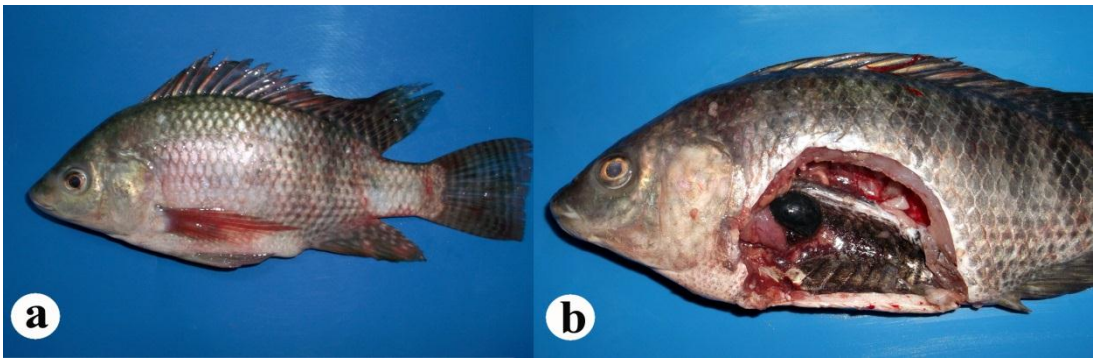

Figure 2: (a)- *O. niloticus* showing haemorrhagic patches on the external body surface, skin erosions, ascites and protruded anal opening. (b)- *O. niloticus* showing swollen gall bladder and congested kidney.

Bacteriological examination

Total number of 18 bacterial isolates was retrieved from 29 infected fish. Pure cultures of *Vibrio anguillarum* (11 isolates) and *Aeromonas hydrophila* (7 isolates) were isolated from succumbed fishes within

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affected fish ponds table 2. The full phenotypic and biochemical characteristics of recovered bacterial isolates are illustrated in table 3

Table 2: Bacterial infections recorded in examined *Oreochromis niloticus* specimens

No. Examined fish	No. Infected fish	Organs	*Aeromonas hydrophila* isolates (7)	*Vibrio anguillarum* isolates (11)
100	29	gills	2	1
		liver	4	5
		kidney	1	4
		spleen	-	1

Table 3: Phenotypic and biochemical characteristics of bacterial isolates retrieved from *Oreochromis niloticus* .

	Aeromonas hydrophila isolates	*Vibrio anguillarum* isolates
Gram- staining	**Gram-negative short bacilli**	**Gram-negative, straight to slightly long curved rods.**
O/129 sensitivity (150 mg)	-	+
B –Galactosidase production (OPNG)	+	+
Arginine dihydrolase production (ADH)	+	+
Lysine decarboxylase production(LDC)	-	-
Ornithine decarboxylase production(ODC)	-	-
Citrate utilization (CIT)	-	Variable
H2S production(H2S)	-	-
Urease production(URE)	-	-
Tryptophane deaminase production (TDA)	-	-
Indole production(IND)	+	+
Acetoin production(VP)	+	+
Gelatinase production(CEL)	+	+
Acid from glucose(GLU)	+	+
Acid from manitol(MAN)	+	+
Acid from inositol(INO)	-	-
Acid from Sorbitol(SOR)	-	+
Acid from rhamnose(RHA)	+	-
Acid from sucrose(SAC)	+	+
Acid from from melibiose(MEL)	-	-
Acid from amygdalin (AMY)	Variable	-
Acid from arabinose (ARA)	Variable	Variable
Cytochrome oxidase prod(OX)	+	+

Histopathological lesions

Histopathological examination of recovered tissue specimens revealed that, hepatopancreas showed diffuse vacuolar degeneration progressed into hepatocytic and pancreatic acinar cell necrosis associated with congestion of main blood vessels and sinusoidal spaces (Fig 3a, b). Posterior kidney on the other hand, showed multifocal tubular vacuolation and necrosis, intra-tubular cast formation with interstitial congestion and activation of melanomacrophage centers (Fig 3e). Spleen also showed multifocal necrotic areas replaced by empty spaces with the presence of stained bacterial clusters neighbored by activated melanomacrophage centers (Fig 3f). Meanwhile gills showed degenerative changes such as separation in-between the epithelial cell lining of the secondary gill lamellae and the underlying capillary bed, hyperplasia with spongiosis of malpighian cells and lamellar fusion with some circulatory changes like congestion and telangiectasis (Fig 3c, d).

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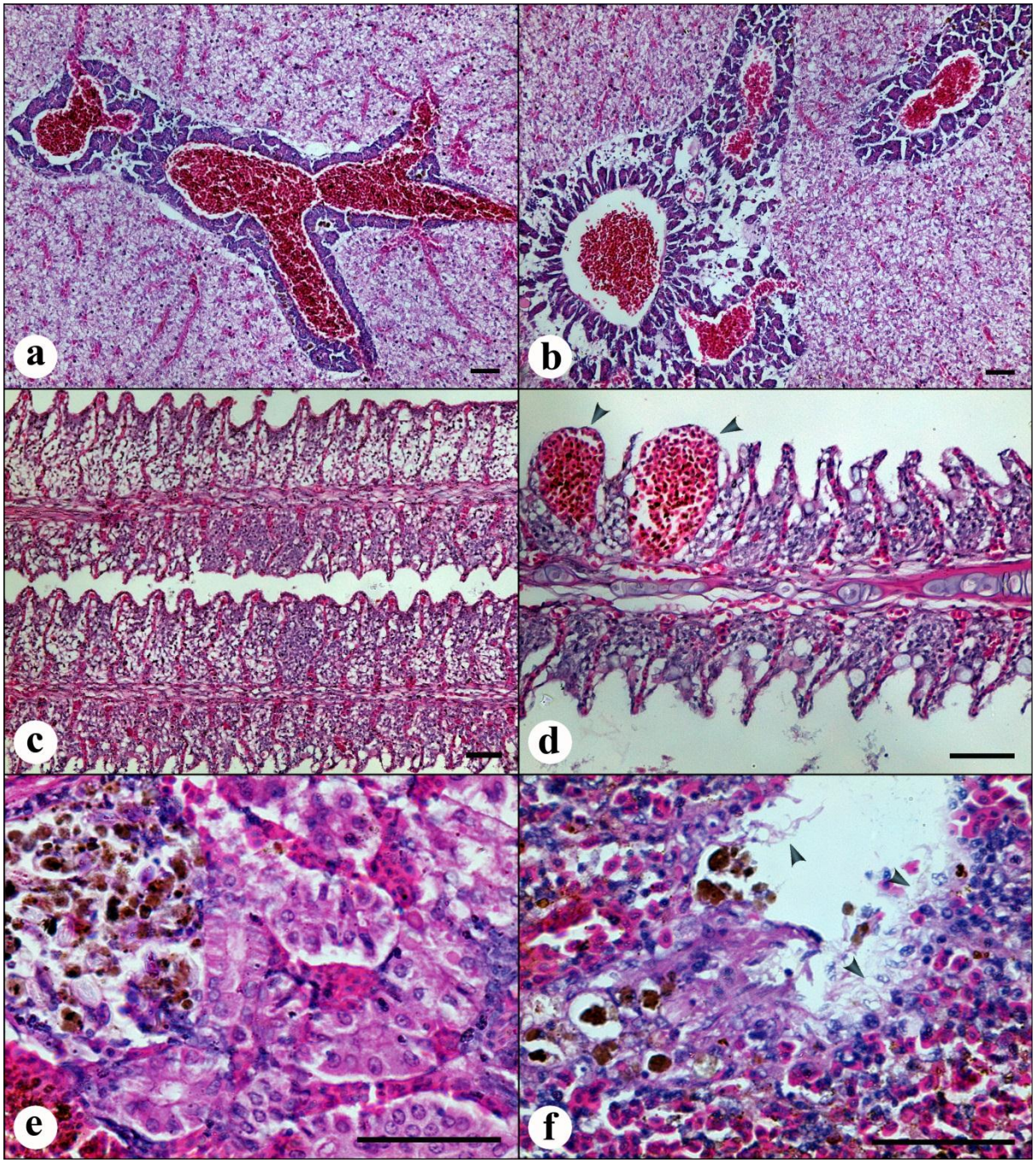

Figure 3: (a) Hepatopancreas of *O. niloticus* collected from the outbreak showing severe congestion of main blood vessels and sinusoidal spaces with diffuse vacuolar degeneration. (H&E, bar= 50 µm). (b) Hepatopancreas of *O. niloticus* collected from the outbreak showing congestion of main blood vessels and sinusoidal spaces with diffuse vacuolar degeneration and hepatocytic and pancreatic acinar cell necrosis. (H&E, bar= 50 µm). (c) Gills of *O. niloticus* collected from the outbreak showing separation in-between the epithelial cell lining of the secondary gill lamellae and the underlying capillary bed with serious hyperplasia and spongiosis of the epithelial lining at the base of the secondary gill lamellae. (H&E, bar= 50 µm). (d) Gills of *O. niloticus* collected from the outbreak showing congestion and telangiectasis (arrow heads) of the secondary gill lamellae. (H&E, bar= 50 µm). (e) Posterior kidney of *O. niloticus* collected from the outbreak showing congestion and discrete tubular vacuolation and necrosis with activation of melanomacrophage centers. (H&E, bar= 50 µm). (f) Spleen of *O. niloticus* collected from the outbreak showing multifocal necrotic areas with presence of bacterial colonies (arrow heads) neighbored by activated melanomacrophage centers. (H&E, bar= 50 µm).

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DISCUSSION

Several environmental stressors can impact the survival and physiology of aquatic animals. Fish can sustain slight fluctuations in water quality measures, but sharp variations adversely affect fish health [1, 4, 16, 17]

The synergy among numerous stressors has detrimental effects on fish greater than individual stressor do since the magnitude of injuries is exaggerated [18**,** 19]. Temperature, dissolved oxygen, pH and ammonia are among the most critical water quality measures potentially impact cultured fishes [1**].** In this sense it is worth to mention that environmental temperature has long been considered the master abiotic factor which systematizes numerous behavioral and physiological aspects of fish [20].

The majority of fish species have their optimal temperature range where functions of their immune system are maximized. Temperature on either side of the optimum level potentially stresses fish [21, 22, 23]. Low water temperatures noticed in fish farms during cold winters have deleterious effects on fish health and survival [1]. Osmoregulatory disturbances and immune-suppression could be the ultimate outcome of long exposure to such unfavorable conditions [19, 22, 24].

Overwinter mortality has critical impacts on the abundance and distribution of many freshwater fishes [25, 26]. Low water temperature exacerbates fish health since several physiological processes are inhibited or reduced [27]. Exposure to long winters and extremely low temperature as typical to that recorded in Barsiq farm (average 5.2°C) has been reported to have immense negative effects on production and survival of tilapia and other fishes [26, 28]. The optimal growth of most tilapia species achieved at water temperature between 25°C and 28 °C [29]. Furthermore, reproduction is impeded at 22 °C while feeding stops below 20 °**C** [30, 31]. Actually, the majority of *Oreochromis* spp. cannot tolerate water temperature below 10-15 °C [32]**.** Mortalities recorded in the Egyptian strain of *O. niloticus* at 13°C to 10 °C [32, 33]. On the other hand, some studies reported slightly better cold tolerance for this strain at water temperature between 11 °C and 7.4 °C [9].

Several factors can influence overwinter mortality rates. Water depth is one of the most critical issues, especially during the cold season [28]. The growth rate and feed conversion of tilapia exacerbate in shallow water ponds (50 cm in depth) concurrently, mortality rates, maximize at such harsh conditions [28] similar to conditions recorded in Barsiq farm since mortality rates approached 98 % in ponds with 50 cm water depth while mortalities decreased in others (100- 150 cm) in depth. This may be justified that fish avoid low water temperature by moving to the bottom, hence, mortalities are reduced**.**

The vertical migration and inshore/offshore movements of tilapia in response to water temperature and depth have been well documented [34, 35]. Fishes live naturally in deeper water during the cold season and migrate to shallower water when the water warms up [28]. Water depth of 1.5 m is used for spawning tilapias, whereas 2-2.5 m depth is recommended for their rearing and wintering [36]. On the other hand, [28] recommended a depth not less than 100-200 cm for tilapia to minimize cold stress and mortality. Moreover authors attributed winter mortalities to the decrease in body protein and lipid at such unfavorable environment (cold water and low depth) as an energy supply to meet the increasing physiological demands.

Mortalities were severe in young-aged tilapia, maximum 40 g, (95 %) compared to adults, more than 150 g, (65 - 70 %) since the existence of size-dependent over-winter mortality has been reported for many fish species, with smaller individuals being more vulnerable than larger one [37, 38, 39].

Results also demonstrated existence of unfavorable oxygen and ammonia levels, 3.5 and 1.03 mg/L respectively. The synergistic effects of unfavorable and abrupt changes in multi-aquatic environmental measures are proved to be sufficiently threatening to fish survival [1, 16, 17]**.**

Prolonged exposure to hypoxic waters can be challenging for fish. Furthermore, unfavorable low dissolved oxygen levels combined with higher ammonia synergize together with other viable components present in these aquaculture facilities to produce eminent cases of fish mortalities [1,19].

Achieved results indicated the possible involvement of some metals in triggering such cases of mortalities. The recorded levels of Cobalt, Copper, Iron, lead, Manganese, Nickel , Zinc, Chromium and Cadmium were; 0.52ppm, 0.02 ppm; 2.16 ppm, 0.48 ppm, 0.24 ppm,0.87 ppm, 0.37 ppm, 0.04 ppm,0.14 ppm respectively in sequential. According to [40] the fresh high reliability trigger value recommended for these metals is; Cobalt 30 µg/L, Copper 1.4 µg/L, Iron 300 µg/L, Lead *3.4* µg/L; Manganese 1700 µg/L ; Nickel 11 µg/L; Zinc 8 µg/L; Chromium 1 µg/L; Cadmium 0.2 µg/L respectively. Moreover, according to Egyptian governmental law, the level of metals in freshwater resources should not exceed the permissible limits; lead 0.05 mg/L; Nickel 0.1 mg/ L; chromium 0.05 mg/ L; cadmium 0.01 mg/ L and 1 mg/ L for (copper, iron and Zinc).

Polluted water weakens the fish host defenses allowing increased opportunities for epizootic disease processes to affect fish populations [1]. Fish collected from polluted environments are more vulnerable to variety of microbial infections than those from non-contaminated habitats a result of immuno-dysfunction [4, 41].

Iron availability potentiates the virulence of numerous bacterial fish pathogens, vibrios, are in the foremost of these devastating microorganisms [1, 42]. Moreover some metals are nutritious and cause eutrophication flourishing bacterial and algal growth resulting in critical deficiencies of oxygen [17]. The combined effects of disturbances in the environmental measures definitely justify tilapia mortalities. Impaired immune mechanisms triggered by theses hostile conditions are strongly accused for establishment of many fish bacterial infections [16, 17, 19]. This may in part justify the recorded *V. anguillarum* and *A. hydrophila* infections in succumbed fishes.

The presences of pathogens alone in culture environments are not always sufficient to cause disease or dysfunction. Other factors regulate the transition from simply harboring a pathogen to actual disease or dysfunction. Unfavorable and Polluted aquatic environments are considerable factors that trigger array of infectious diseases of fish through immuno-suppression [1, 41].

It is also pertinent to denote that drop in temperature causes cold-induced fasting, thermal stress and metabolic depression. Furthermore, Low temperature is selectively suppressive to T cell responses and antibody synthesis [43, 44]. The end outcome is a lower immune capacity rendering fish more susceptible to bacterial infections [45]. Noticeable mortalities have been repeatedly recorded during such periods [1, 4, 46,47]. Furthermore, virulence of several fish pathogenic agents is exacerbated during such adverse conditions [1, 48].

Regarding histopathological lesions noticed in this study, circulatory, degenerative, proliferative and necrotic alterations were distinctive. Devastation of the vital components of the circulatory and immune system by bacterial extracellular products is thought to be the corner stone behind these pathological alterations. Haemolysins and Proteases are at the forefront and extensively contribute to the grievous nature of these pathogenic agents [4,23 ,49] .

CONCLUSION

Extremely low water temperature and long winters have immense deleterious effects on *O. niloticus* survival. The degree of tolerance to such critical conditions is dependent upon environmental effects. Water depth is one of the most critical issues that can influence overwinter mortality. Furthermore, synergistic effects of unfavorable and abrupt changes in multi-aquatic environmental measures definitely threaten fish survival. Fish can sustain slight fluctuations but sharp variations adversely affect fish health. Impaired immune mechanisms triggered by theses hostile conditions are strongly accused for establishment of many fish bacterial infections.

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