

SAPROLEGNIOSIS IN FRESHWATER CULTURED TILAPIA NILOTICA (*ORECHROMIS NILOTICUS*) AND TRIAL FOR CONTROL BY USING BAFRY D50/500

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Abstract

This study was carried out on 100 cultured *Tilapia nilotica* in floating cages with high stocking density suffered from saprolegniosis, which considered the most important causes of economic losses in freshwater fish second to bacterial disease in Egypt. The fish were subjected to full clinical, postmortem, bacteriological and histopathological examination, also trial for control using Bafry D50/500 and hematological examination of treated fish. Clinically, the infected fish showed loss of equilibrium, lethargy, unable to feed, hemorrhage at the base of fins may extended to cover all the body surface, the main characteristic lesions of saprolegniosis was appearance of cotton wool like tufts on the fins (dorsal, caudal, and pectoral fins); also, on eyes, the head and mouth and uni or bilateral blindness ended with death. Postmortemally, the infected fish showed pale grayish gills, the intestine were free from any food particles, dark enlarged liver, distended gallbladder and splenomegaly. Bacteriologically, *Aeromonas* Spp. and *Pseudomonas* Spp. were isolated from infected fish suffered from saprolegniosis. The wet mount from infected lesions showed the presence of large non septated hyphae, the colonies on SDA started with cysts of long hairs with cottony color, and then became gray, then black color colonies. From the microscopical and morphological finding were characteristic for saprolegnia. *Saprolegnia parasitica* were isolated with higher percentage from skin followed with fins, eyes, and mouth, but not isolated from liver nor kidney. Trial of using Bafry D50/500 for control in saprolegniosis in a dose of 0.75 ml/L (375ppm) for 10-12 minutes for 3 successive days as a bath gave a good results in treatment of the diseased fish in aquaria, this were indicated the rate of mortality reach 16.7% without any effect on the healthy stat of treated fish and also no effect on hematology and blood chemistry of treated fish with Bafry D50/500. Histopathological finding of the infected fish showed epithelial desquamation of the epidermis, erosion and ulceration of the infected area, edema, hyaline degeneration, and aggregation of M.M.C. and fragment of fungal hyphae occurred in the underling dermis of the skin, muscles; The gills of the infected fish showed complete desquamation of majority secondary lamellae, and gill arch was edematous with presence of aggregation of fungal spores and M.M.C., Histopathological finding of treated fish with Bafry D50/500: skin showed increase of M.M.C. and slight sloughing of most superficial layer of epidermis but the gills showed increasing and hyperplasia of epithelial cells covering of primary lamellae, this indicate that the infected fish was treated with BafryD50/500.

Key word: *Tilapia*, Saprolegniosis, Bacteria, Histopathology, Bafry D50/500

INTRODUCTION

Fishes are the primary source of protein for human in many areas of the world and this is also in Egypt. Outbreaks of water born fungal infections of fish, Amphibians, and Reptiles are common problems especially in fish farms and hatcheries. Of particular concern is saprolegniosis, which is an infectious fungal disease that is wide spread in all stages of the life cycle of fish. Saprolegniosis infection may contribute to heavy mortality among fishes and are widespread in freshwater fish's ecosystem and affect wild and cultured fishes, also involving living, dead and eggs (Noga, 1996-Bruno and Wood, 1999-Hussein and Hatai, 1999-and Hussein *et al*, 2001). Saprolegniosis considered as single largest cause of economic losses in aquaculture (Meyer, 1991) second only to bacterial disease in economic importance. In Egypt, the mycotic disease constitute one of the most important disease causing troubles in fresh culture with several economical losses especially saprolegniosis (Easa, 1984, Shaheen ,1986, and El Zayat,1988).Saprolegnia considered agent of secondary infection arising from conditions as bacterial infections, poor husbandry including poor water quality, adverse water temperature , all of these factors increased occurrence of saprolegnia infections (Pickering and Willoughby,1982, and Bailey,1984). Saprolegniosis are generally restricted to chronic, steady losses(Bruno and Wood,1999-Pickering and Willoughby,1982) and cause 50% mortality in Salmon fish (Hatai and Hoshiai,1994), 50%losses in *Anguilla anguilla* (Bruono and Wood,1999), 50%losses in channel catfish in USA (Bruono and Wood,1999), and affect tilapia (*Oreochromis niloticus and Oreochromis mossambicus*) in hatchery and fingerlings stages (Easa and Amin,1987,Ogbonna,1989, Aly *et al.*, 1996, Aly and El Ashram,2000).The seasonal variation play an important role in spreading of the saprolegnia infections in freshwater fish especially during late autumn, common in winter months and early spring where the temperature was low (Hughes,1962) .Much work has been devoted to the examination of various types of fish fungicides and their effects either on aquatic animals or on the environment. Malachite green and formalin are the most potent fish fungicides although they have an acute impact on the aquatic ecosystems (Willoughby and Roberts, 1992, Schreier *et al.*, 1996). Other problems with theses substances include an immune suppressive effect on repeatedly treated fish (Prost and Spinska1989), terratogenic and mutagenic effect due to hazardous residues in fish tissues (Meyer and Jorgenson, 1983, Fitzpatrick *et al.*, 1995, Meinert *et al.*, 1995, and Bruono and Wood, 1999).Formalin, solution 37% formaldehyde is effective in treating saprolegnia but has effect on both environments personal who handle it (Fitzpatrick *et al.*, 1995). For these reasons many researches have been investigating the use of safer compounds that have no

harmful effect on fish and their eggs or on ecosystem (Hussein *et al.*, 2001). So, the use of hydrogen peroxide (H₂O₂) is a potential chemotherapeutant compound looked upon aquaculture community with increasing interest. The compound is considered environmentally compatible as well as being an effective treatment for a variety of external fish disease, fungicide, bactericide, parasiticide on fish and fish eggs, (Marking *et al.*, 1994, Mc Andrew *et al.*, 1998, Derksen *et al.*, 1999, Hawe *et al.* 1999, Schreier *et al.*, 1996 and Speare & Arsenault, 1997). Hydrogen peroxide has a low regulatory priority classification by United States food and drug administration (FDA) for use as fungicide on fish and fish eggs (Schnick 1994). The apparent lack of adverse environmental impact of hydrogen peroxide combined with its effectiveness as an antimicrobial agent (Rach *et al.*, 1997). Therefore, the current investigation was planned to focus on saprolegniosis as a major fungal diseases affect freshwater fish (wild and cultured fishes)especially tilapia species as well as the optimal preventive trials to control saprolegniosis by using chemicals safe to both environment and personal who contact with these chemicals as Bafry D50/500 .

MATERIALS AND METHODS

I - Materials

I.1 Naturally infected fish

A total number of 100 *Oreochromis niloticus* fish showed skin lesions were collected from floating cages in El Bostan Damietta during the period November 2005 and January 2006, with average 75 ± 5 gm body weight and length 13 ± 2 cm. the collected fish were transported in ice box to the Central Laboratory for Aquaculture (CLAR) Abbassa Abou Hammad Sharkia, Egypt subjected to full clinical, bacteriological and mycological investigation.

I.2 Fish for experiment

A total number of 210 healthy fish with average body weight 70 ± 5 gm *Oreochromis niloticus* were collected from earthen pond of (CLAR) and transported to the wet laboratory and acclimatized to the conditions for 2 weeks, maintained at 25 ± 2 °C in glass aquaria of 40 X 50 X 80 cm, supplied with chlorine free water, the temperature was thermostatically adjusted at 25 ± 2 °C and fish were feed with 30% protein according to their body weight.

I.3 Media

Sabouraud dextrose agar (SDA) (Adwic SCG) was used for isolation of fungus and was prepared by dissolving 65 gm /liter of distilled water by gentle heating and sterilized in autoclave at 121 °C for 15 mint and chloramphenicol was added by 50 mg / ml (Cruickshank *et al.*, 1975).

I.4 Chemicals used for treatment

a- Bafry D 50/500(H₂O₂+Silver ions) FMT: 106 Makram Obeidst., Nasr City, Cairo, Egypt.

I.5 Stain

Lacto phenol cotton blue stain was used (Leonor and Carey, 1978)

II - Methods

II-1 Clinical examination:-

One hundred naturally infected living fish were examined for abnormal behaviors and external lesions on the skin, eye, and gills according to the method described by Amlacker, 1970.

II – 2- Postmortem examination

Postmortem examination was done on living or freshly dead fish and examination of internal organs was done according to Amlacker, 1970, and Lucky, 1977, to show any abnormalities.

II -3-Bacteriological examination

Samples were taken from skin, gills and internal organs of infected fish and streaked on tryptic soy agar and incubated at 27 °C for 24-48 hrs, then purification and identification of isolated bacteria was done according to Austin and Austin (1993).

II -4- Mycological examination

II -4-a: Isolation of fungi:- was carried out from naturally infected fish, samples were taken from fish showing skin lesions, eye, fins, gills, mouth, spleen, liver and kidney lesions were collected and inoculated onto SDA medium plates and incubated at 20 ± 2°C for 3-4 days ,subculture on the same media was done for purification.

II -4- b: Identification of the isolates

All positive cultures were examined for colonial growth, morphological features and microscopical characteristics.

The morphological features include appearance of the cultures, rate of growth, texture of the surface colonies, colonies color according to Refai and Al Doory, 1986.

Microscopical examination was done for wet preparations of the skin lesions and mycelia cultured on (SDA) to detect septation of hyphae according to Dvorak and Otcerasek, 1969.

II -5: Biochemical analysis

Blood samples were taken from caudal vein under anathematic by using MS222 (200mg / L) to study the effect of bafry D50/500 on the blood chemistry parameters of treated *O. niloticus* according to Wotton and Freeman (1982).

II -6: Histopathological examination of infected and treated fish

Samples taken from skin, fins, and gills from infected and treated fish, fixed in neutral buffered formalin, embedded in paraffin wax, sectioned in 5.0 microns and stained with Hematoxylin and Eosin (H&E). (Roberts, 2001)

Experimental design

Experiment 1

The experiment was carried in wet laboratory by using 15 aquaria for (5) groups, three replicate for each group as shown in Table (1) each replicate contain 10 fish.

Experiment II - (safety test)

This experiment was carried out after experiment I, 90 healthy *O. niloticus* were used in three groups, C1: (Control without treatment), C2 (control treated with Bafry 0.75 ml / L, 375ppm) as bath for 10-12 minutes as shown in Table(2) to study the effect of best concentrations of both drugs on condition and behavior of healthy fish.

Table 1. Application of Bafry D50/500

Group	Treatment	Concentration	Mode of using	Time of exposure	Duration
1	Control infected non treated	–	–	–	3 successive days
2	Control healthy non treated	–	–	–	3 successive days
3	Treated with Bafry D50/500 0.25ml/L(125ppm)	0.25ml/L (125ppm)	bath	10-12 minutes	3 successive days
4	Treated with Bafry D50/500 0.5ml/L(250ppm)	0.5ml/L (250ppm)	bath	10-12 minutes	3 successive days
5	Treated with Bafry D50/500 0.75ml/L(375ppm)	0.75ml/L (375ppm)	bath	10-12 minutes	3 successive days

Table 2. safety test = tolerance test

Group	Treatment	Concentration	Mode of using	Time of exposure	Duration
C1	Control healthy without treatment	–	–	–	3 successive days
C2	Control healthy Treated with Bafry D50/500 0.75ml/L(375ppm)	0.75ml/L (375ppm)	bath	10-12 minutes	3 successive days

RESULTS

► Results of clinical examination

The main clinical signs appeared on the infected fish were lethargy, loss of equilibrium, unable to feed, hemorrhage at the base of the fins, sometimes, the hemorrhages extended to cover all the body surface, as showing in photo(1),(2), the main characteristic lesions of saprolegniosis was appearance of cotton wool like tufts on the dorsal, tail (caudal), pectoral fins also appearance on the eye, head and mouth of fish as shown in photo.(3),(4) unilateral cloudy or opacity of the eye ended by blindness, emaciation and death occurred, as shown in photo(5).

► Results of postmortem examination

The main postmortem lesions are appearance of cotton wool tufts on caudal fin(tail), pale to grayish gills, serious fluid or exudates in the abdominal cavity, intestine free from any food particles, dark enlarged liver, distended gall bladder with bile, splenomegaly and congested kidney as shown in photo(6).

► Results of bacteriological examination

The bacteriological examination showed that isolation of Gram negative bacteria and identified as *Aeromonas species* and *Pseudomonas species* fro fish suffered saprolegniosis.

► Results of mycological examination.

The results of mycological examination showed that isolation of 238 isolates from 640 samples from 100 infected *Oreochromis niloticus* sampled from skin, gills, fins, eye, kidneys and mouth as shown in Table (3).

The positive colonies on (SDA)at 20°Cfor 3-4 days started with cysts of long hairs with white cottony color after that became grey then black after 96 hrs. incubation, as shown in photo (7).

The wet preparation of skin, gills, eye, and mouth lesions showed masses of mature and immature sporangia filled with large number of sporangiospores, the hyphae appeared profusely branched and were non septated, these morphological findings were characteristic of the saprolegnia species as shown in photo(8).

Table 3. Incidence of *saprolegnia parasitica* isolated from different organs and tissues

Fish species	organs	skin	fins	gills	eyes	mouth	liver	kidney	spleen	Total
100 diseased <i>O.niloticus</i>	No. of samples	100	100	100	25	15	100	100	100	640
	No. of isolates	85	78	35	25	15	0	0	0	238
	%	35.71	32.77	14.70	10.50	6.30	0	0	0	100%

As shown in Table (3) the *saprolegnia parasitica* isolated from skin and fins with higher percentage (35.71% and 32.77%) respectively followed by gills, eye, and mouth (14.70%, 10.50%, 6.30%) respectively.

Table 4. Results of application of Bafry D 50/500 in disappearance of saprolegnial fungal growth and signs.

group	No of fish	Follow up through 10 days		Fungal growth and clinical signs	Survival rate %
		Dead	Survive		
1	30	30	0	+++	0 %
2	30	0	30	---	100 %
3	30	22	8	++/-	26.6 %
4	30	16	14	++/--	46.3 %
5	30	5	25	---	83.3 %
C1-9	30	0	30	Healthy fishes	100 %
C2-10	30	0	30	Healthy fishes	100 %

+: signs still occur - : signs disappear

As shown in Table (4) the Bafry D50/500 gave a good result in the treatment and disappearance of fungal growth and clinical signs in affected fish in aquaria especially in a dose of 0.75 ml/L(375ppm) as a bath for 10-12 minutes for 3 successive days and the mortality rate reach 16.7% and the survival rate reach 83.3% with no effect on healthy state of treated fish, this indicated with no significance in blood parameter and chemistry of treated healthy fish and non treated healthy fish except increasing in glucose level and AST as shown in Table(5)

Table 5. Results of effect of Bafry D 50/500 on hematological parameters and blood chemistry.

Treatment	PCV	Hb	Total protein	Albumin	Globulin	A/G	Ca++	glucose	ALT	AST
C1	26.5 ±1.4	5.2 ±0.15	4.5 ±0.21	1.41 ±0.15	3.09 ±0.18	0.456 ±0.021	7.6 ±0.52	66 ±3.3	32 ±4.2	75 ±4.5
	a	a	a	a	a	a	b	b	a	b
C2	26.3 ±1.5	5.00 ±0.12	4.3 ±0.18	1.39 ±0.12	2.91 ±0.12	0.477 ±0.023	7.5 ±0.55	98 ±6.3	40 ±3.8	114 ±8.5
	a	a	a	a	a	a	b	a	a	a

► Results of histopathological examination of naturally infected fish

The skin showed epithelial desquamation in the epidermis which displayed either erosion to ulceration in the infected area. The other epidermal cells suffered vacuolar degeneration and focal necrosis. The underlying dermis was edematous and contained fragments from the fungal hyphae with focal aggregation of melanomacrophages cells. The underlying muscles exhibited intramuscular edema, hyaline degeneration and Zinker's necrosis. The necrotic muscles infiltrated with numerous mononuclear leukocytes and some melanomacrophages. *Saprolegnia* spp, appeared by H&E stain as non septated hyphae of variable size and lengths. As shown in photo (9). The gills showed desquamation to the majority of the secondary lamellae with few mononuclear leukocytes infiltrated the primary lamellae. The gill arch was edematous and contained the fungal spores and hyphae as well as some melanomacrophages and mononuclear cells. As shown in photo (10).

► Results of histopathological examination of treated fish

Skin of *Tilapia nilotica* (*O.niloticus*) treated with BafryD50/500 showed increase of M.M.C and slight sloughing of most superficial layer of epidermis. As shown in photo (11). Gill of *Tilapia nilotica* (*O.niloticus*) treated with BafryD50/500 showed hyperplasia of epithelial cells covering of primary lamellae. As shown in photo (12).

DISCUSSION

In Egypt the mycotic disease constitute one of the most important diseases causing troubles in fresh culture with several losses especially saprolegniosis Shaheen, 1986 and El Zayat, 1988. Saprolegniosis infection may contribute to heavy mortality among fish and are wide spread in freshwaters ecosystem and affect wild and cultured fishes ,also involving both living ,dead and eggs and was considered as single largest cause of economic losses in aquaculture, second only to bacterial disease in economic importance, Meyer,1991, Noga,1996, Bruno and Wood,1999, Hussein et al.,2001. Saprolegniosis considered as localized infection not systemic infection, generally are external and appear any where over the body surface especially fins, eyes, gills and ulcerated area on the body, the clinical signs appear on the fish suffered from saprolegniosis were developed of grayish white cotton like tufts on fins(dorsal, caudal and pectoral fins) also on eyes and mouth, emaciation and death occurred due to blindness and the affected fish unable to feed., also respectively distress occur due to fungal growth on gills so, the gills become very pale due to the excessive mucus secretion and the fungal growth. In some cases, the affected fish showing muscle lesions as erythema and ulceration due to the lytic action of bacteria, so saprolegniosis considered as secondary invader to the bacteria, these results

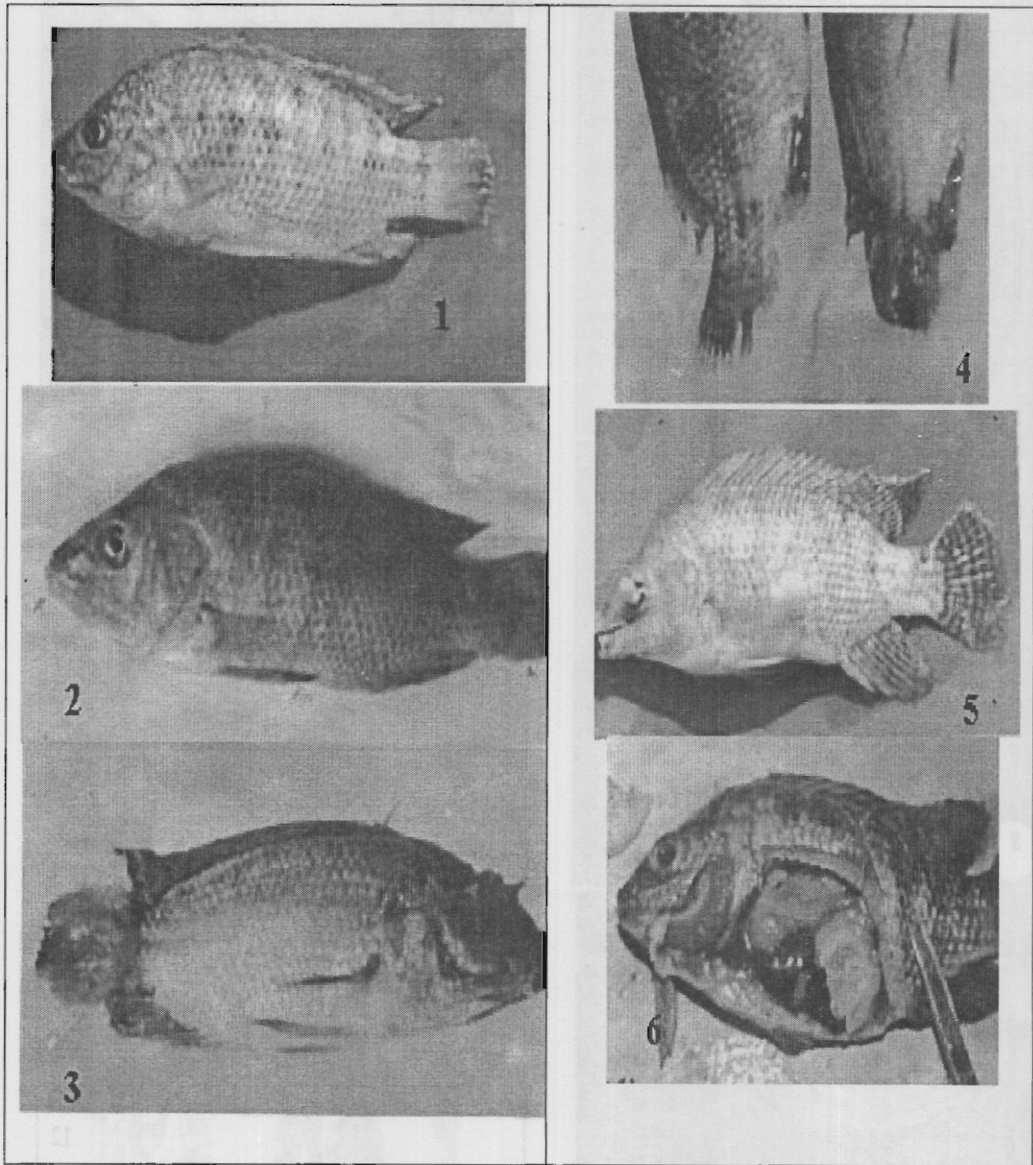
recorded also by Aly *et al.*,1996,Noga,1996,Bruno and Wood,1999,Hussein and Hatai,1999, Aly and El Ashram,2000,Hussein *et al.*, 2001, and El Ashram *et al.*,2007.The main postmortem lesions appear were pale gills due to respiratory distress and excessive mucus secretion, this results observed by Plumo,1994, the enlarged liver, kidney, spleen and gallbladder may be due to systemic bacterial infection and not attributed to saprolegniosis, these results agree with Paperna *et al.*,1983,and Bailey,1984. Saprolegnia considered being secondary invader accompanied by opportunistic bacteria. The results of bacterial examination showed isolation of gram negative bacteria and were identified as *Aeromonas species* and *Pseudomonas species*, these results observed by Paperna *et al.*, 1983, Bailey, 1984, Aly and El Ashram, 2000, and El Ashram *et al.*, 2007. The results of mycological examination showed that isolation of *Saprolegnia parasitica* which was branched non septated tubular hyphae and isolated with higher percentage from skin (35.71%), followed by fins(32.77%), gills(14.70%), then mouth(6.30%) and not isolated from liver, kidney and spleen, these results agree with Marzouk *et al.*,1990 and Marzouk *et al.*, 2003. The results of application of Bafry D50/500 for control of *Saprolegnia parasitica* revealed that using of Bafry D50/500 in a concentration of 0.75ml/L (375ppm) as a bath for10-12 minutes is the best concentration for elimination of saprolegnial growth with no affect on behavior of treated fish and also on healthy fish. Bafry D 50/500 is a mixture of hydrogen peroxide which is an oxidizing agent ant silver ions which is are added for long term efficiency, these results recorded by Marking *et al.*, 1994, MC Andrew *et al.*, 1998, Derksn *et al.*, 1999 and Hawe *et al.*, 1999. The results of effect of best concentration of Bafry D50/500 on blood parameters showed that no significance difference between control healthy non treated and the healthy treated fish , except glucose which is higher in healthy treated than non treated one due to H₂O₂ affect on cortisol level in the blood, also, AST is higher in treated healthy fish than healthy non treated, these results accepted with Tort *et al.*, 2003.The results of histopathological examination of skin showed epithelial desquamation in the epidermis and other epidermal cells suffered vacuolar degeneration and focal degeneration thus due to sever spongiosis. Skin showed epithelial desquamation in the epidermis which displayed either erosion to ulceration in the infected area. The other epidermal cells suffered vacuolar degeneration and focal necrosis. The underling dermis was edematous and contained fragments from the fungal hyphae with focal aggregation of melanomacrophages cells. The underlying muscles exhibited intramuscular edema, hyaline degeneration and Zinker's necrosis. The necrotic muscles infiltrated with numerous mononuclear leukocytes and some melanomacrophages. Saprolegnia spp, appeared by H&E stain as non septated hyphae of variable size and lengths. As shown

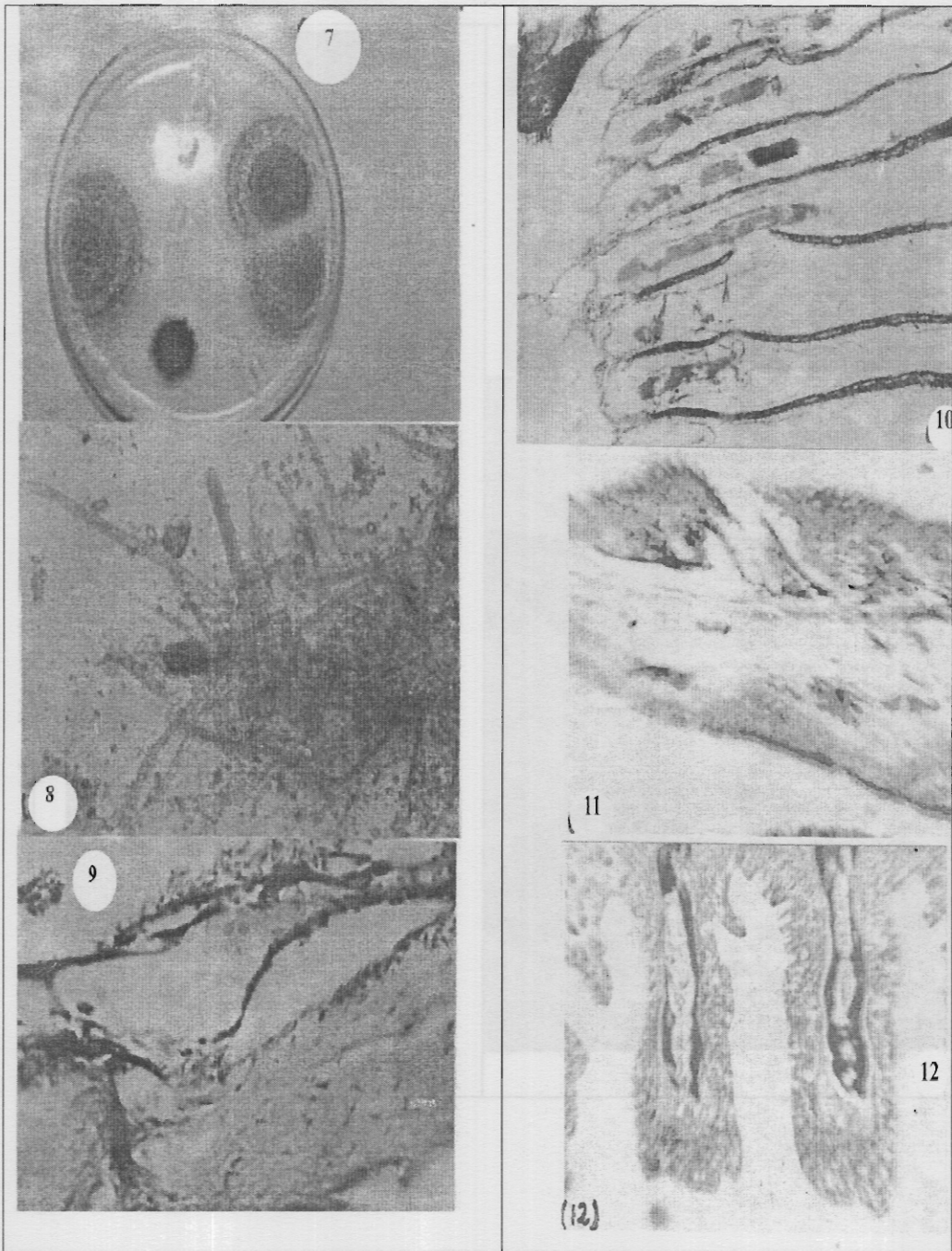
in photo (9). The gills showed desquamation to the majority of the secondary lamellae with few mononuclear leukocytes infiltrated the primary lamellae. The gill arch was edematous and contained the fungal spores and hyphae as well as some melanomacrophages and mononuclear cells. As shown in photo (10), were similar to those observed by Amin *et al.*, 1985, Ferguson, 1989, Aly *et al.*, 1986, Aly and El Ashram, 2000, El genaigy *et al.*, 2004, and El Ashram, 2007. The results of histopathological examination of treated fish with Bafry D 50/500 showed increase of MMC and slight sloughing of most superficial layer of epidermis and presence of vaculation indicating the disappearance of causative agent, also, the gills showed hyperplasia or increasing of epithelial cells covering of the primary lamellae indicating slight healing of the infected gills of treated fish.

CONCLUSION

The most effective method for controlling and preventing saprolegniosis in fish ecosystem is a combination of good fish management as good water quality and avoid adverse water temperature and Bafry D50/500 used for controlling in a concentration of 0.75ml/L (375PPm) as a bath for 10-12 minutes, due to Bafry D50/500 is a mixture of hydrogen peroxide and silver ions, hydrogen peroxide considered as fungicidal, bactericidal and parasiticidal effect as mentioned with Marking *et al.*, 1994, Mc Andrew *et al.*, 1998, Derksen *et al.*, 1999, Hawe *et al.*, 1999, Schreier *et al.*, 1996 and Speare and Ar senault, 1997. Also hydrogen peroxide has no effect on healthy condition and blood parameters of treated fish, Tort *et al.*, 2003. Silver ions added for long term efficiency and react as a catalyst.

Table NO.	Title
1	Application of Bafry D50/500
2	Safety test = tolerance test
3	Incidence of <i>saprolegnia parasitica</i> isolated from different organs and tissues
4	Results of application of Bafry D 50/500 in disappearance of saprolegnial fungal growth and signs.
5	Results of effect of Bafry D 50/500 on hematological parameters and blood chemistry.





List of photo

Photo No.	Description
1&2	Hemorrhage at the base of the fins, and extended to cover all the body surface
3&4	Cotton wool like tufts on the dorsal, tail (caudal), pectoral fins also appearance on the eye, head and mouth of fish
5	Unilateral cloudy and opacity of the eye ended by blindness and emaciation
6	Postmortem lesions are appearance of cotton wool tufts on caudal fin(tail), pale to grayish gills, serious fluid or exudates in the abdominal cavity, dark enlarged liver, distended gall bladder with bile, splenomegaly and congested kidney
7	The positive colonies on (SDA)at 20°Cfor 3-4 days started with cysts of long hairs with white cottony color after that became grey then black after 96 hrs.
8	The wet preparation of skin, gills, eye, and mouth lesions showed masses of mature and immature sporangia filled with large number of sporangiospores, the hyphae appeared profusely branched and were non septated, these morphological findings were characteristic of the saprolegnia species .
9	The skin of infected tilapia, showed epithelial desquamation in the epidermis. The other epidermal cells suffered vacuolar degeneration and focal necrosis. The underling dermis was edematous and contained fragments from the fungal hyphae with focal aggregation of melanomacrophages cells. The necrotic muscles infiltrated with numerous mononuclear leukocytes and some melanomacrophages. Saprolegnia spp, appeared by H&E stain as non septated hyphae of variable size and lengths
10	The gills of infected tilapia, showed desquamation to the majority of the secondary lamellae with few mononuclear leukocytes infiltrated the primary lamellae. The gill arch was edematous and contained the fungal spores and hyphae as well as some melanomacrophages and mononuclear cells
11	Skin of Tilapia nilotica (<i>O.niloticus</i>) treated with BafryD50/500showed increase of M.M.C and slight sloughing of most superficial layer of epidermis
12	Gill of Tilapia nilotica (<i>O.niloticus</i>) treated with Bafry D50/500 showed hyperplasia of epithelial cells covering of primary lamellae.

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