http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 4 (9): 1133-1137, 2001

Microbiological Study on a Cyanobacterium Isolated from Fresh Water Fish Ponds with a Reference to its Hepatotoxic Effect

[1]Mohamed T. Shaaban and [2]Saber A. Sakr
[1]Botany and [2]Zoology Department, Faculty of Science, Menoufiya University, Shebin El-Kom, Egypt

Abstract: The present work deals with identification of a cyanobacterium found to be predominant causing blooms on the water surface in fresh water, fish ponds. The cyanobacterium blooms were found to be specially flourishing in parallel with low fish productivity. *Microcystis aeruginosa* was suspected to some biological studies including, the antibacterial effects of its cell free extract against Gram +ve and Gram -ve bacteria and hepatotoxic effect of the cell free extract on the liver of albino rats. Treatment with a single intraperitoneal dose (½ LC_{50}) of the extract caused elevation of alkaline phosphatase and transaminase (GOT & GPT) in the animal sera. Histological examination of the liver of treated animals revealed congestion of blood vessels and abundance of leucocytic infiltration, a matter which may be due to one or more of microcystins present in this cyanobacterium.

Key words: Cyanobacteria, antibacterial effect, hepatotoxicity, rat

Introduction

Fish productivity in fresh water is correlated to flourishment of phytoplanktonic components, bacteria and algae (Knud-Hansen and Batterson, 1994; Garg and Bahatnagar, 1996). Cyanobacterial blooms have been detected in fresh water ponds and lakes all over the world. Some of these cyanobacteria were found to be the source of some potent toxins (Charmichael, 1988). Cyanobacteria were recorded to represent up to 17% of the phytoplankton investigated in fresh water fish ponds (Shaaban *et al.*, 1999). In spite of great importance of cyanobacteria for aquatic life nutrition, specially for fish growth and productivity many of them were recorded to be highly toxic affecting the growth of aquatic organisms (Turell and Middelbrook, 1988). Toxic water blooms of cyanobacteria have been found flourishing during mid to late summer in fresh water ponds and lakes in U.S.A., Canada, Russia, Europe, S. Africa, S. America, India, Japan, Middle east and Australlia (Charmichael *et al.*, 1985). Among important toxins produced by cyanobacteria are those representing a related family of cyclic hepatopeptides with molecular weights ranging between 909 and 1044 (Botes *et al.*, 1982). These toxins are referred to as microcystins (Charmichael, 1988). Such toxins have been recorded to be responsible for many deaths among liver stock and wild life (Watanabe and Oishi, 1983). The general pathology of animals which have been acutely poisoned by microcystins is confined to the liver (Carbis *et al.*, 1995). The aim of the present work was to identify the most abundant cyanobacterium and to investigate the antimicrobial potency of the cell free extract against the Gram-positive and Gram-negative bacteria and to study the hepatotoxic effects of the cell free extract on liver of albino rats to quantify the extent to which this microorganism is harmful to both animals and microorganisms.

Materials and Methods

Test organism: An unknown cyanobacterium forming blooms on the surface of the fresh water fish ponds (Central Lab. of Aquaculture Research of Abassa-Sharkia-Egypt) was collected during March - April, 1999.

Nutritive media and growth conditions: Cyanobacterian bloom samples were cultivated and enriched on an inorganic medium (Allen, 1968) at constant temperature 30 ^{0}C for about 3 days, under continuous irradiance of 30 μm (photon) m^{-2} s^{-1} provided from 6 incandescent lamps. Also the specific medium for growth of *Microcysts aeruginosa* (A modification of Jansen medium according to Parker *et al.*, 1997) was applied in the identification process and enrichment of the microorganism. Two stock solutions A & B were prepared. The gradients of solution A were in g/L, $NaNO_3$, 67.0, Na_2HPO_4, 0.67; K_2HPO_4 3.8; Tricine buffer, 25.0. The gradients of solution B were in g / L, $MgSO_4$ 10.0, $MgCl_2$ 5.0; $FeCl_3$ 0.5, $CaCl_2$ 4.2; Na EDTA, 4.4 and trace elements. Twenty ml of solution A were combined with 980 ml of glass distilled water, pH adjusted to 8.5 with 1N NaOH and autoclaved, 20 ml of solution B were combined with 980 ml of glass distilled water, autoclaved and then the two solutions were combined 1 : 1 v/v. Samples were examined under a light microscope at x400 and identified according to Prescott (1978).

Preparation of cell free extract: In the third day of growth, the cell suspension in flasks were transferred, immersed and swirled in orbital shaking incubator oscillating at 300 rpm at 25 °C for 24h. The cells were then harvested by centrifugation at 4 °C, washed by deionized water and stored frozen until extraction. Samples were treated in the cold in MSE ultrasonic homogenizer for 5 minutes at intervals of 15-20 sec followed by equal periods of cooling in ice bath. The lysed or disrupted cell mixture was then centrifuged at 15000g for 40 minutes and the supernatant was used in the biological activity tests according to El-Sheekh and Rady (1995).

Antibacterial potency of cell free extract of the cyanobacterium was investigated against Gram +ve *Bacillus subtilis* and the Gram -ve *Pseudomonas aeruginosa*. A series of dilutions were prepared from the cell free extract in water: 1:10 w/v, 1:20 w/v, 1:30 w/v and 1:40 w/v small discs (5mm diameter) were full wetted (saturated) by the extract solution at different dilutions. Discs were then put on the surface of growth solidified nutritive plates inoculated by the test bacteria, incubated at 30 °C. After 24 to 48 hours, the plates were investigated for the presence of clear zones around discs and diameters of inhibition zones were measured in mm (Izzo *et al.*, 1995).

Hepatotoxic study: Adult male albino rats (*Rattus norvegicus*) weighing 230 ± 5g, were kept in laboratory under constant conditions of temperature (24 ± 2 ^{0}C) for at least one week before and throughout the experimental work, being maintained on a standard diet and water was available *ad*

libitum. Animals were divided into two groups. Each rat in 1st group (30 rats) was injected intraperitoneally with one ml of ½ LD_{50} of cell free extract of cyanobacterium as a single dose, while animals of the 2nd group (20 rats) were used as control. For enzyme determination, blood was collected from control and treated animals. Sera were obtained by centrifugation of blood samples and stored at –20 °C. Serum alkaline phosphatase, GOT (glutamate oxaloacetate transaminase) and GPT (glutamate pyruvate transaminase) were measured using a fully automated Hitachi 911 analyzer (Tokyo, Japan). Commercial Randox kits (Randox Laboratories, Ltd. U.K.) were used in this analysis.

For histological study, treated animals and their controls were sacrificed by decapitation and their liver were removed and fixed in Bouin's fluid for 24 hours. After fixation, tissues were dehydrated through ascending grades of ethanol, cleared in xylene and finally embedded in paraffin wax. Specimens were sectioned at 5 microns using a rotary microtome and sections were stained with hematoxylin and eosin. The results were analyzed statistically using student's "t" test (Sendecor and Cochran, 1971).

Results

The cyanobacterium characterized by: (1) being responsible for the development of massive surface blooms in fresh water fish ponds (2) growing on solidified nutritive medium under photo autotrophic conditions forming irregular globular to oval colonies (3) showing cells arranged within the colonial mucilage in microscopic investigation (4) cells seem round purple to brown as a result of refraction of light by the characteristic pseudovacules and (5) being able to grow well on the nutritive medium specific for the growth of *Microcystis aeruginosa* was identified according to Prescott (1978) as *Microcystis aeruginosa* (Fig. 1).

Antimicrobial effect: As seen in Fig. 2 and 3, and Table 1 the cell free extract of cyanobacterium was found to have potent inhibitory effect against Gram +ve bacteria represented by *Bacillus subtilis* and Gram -ve bacteria represented by *Pseudomonas aeruginosa*. Clear inhibition zones with different diameters were observed around discs saturated by cell free extract at different dilutions in water.

Table 1: Mean values of inhibition zone diameters on growth plates of *Pseudomonas aeruginosa* and *Bacillus subtilis* due to different dilutions of cell free extract of *Microcystis aeruginosa*.

Test organism.	Inhibition zone (diameter in mm.)			
	Extract dilution in water w/v.			
	1 : 10	1 : 20	1 : 30	1 : 40
Pseudomonas aeruginosa	34.0±2	24.0±3	19.0±2	10.0±3
Bacillus subtilis	28.0 ±4	18.0±2	15.0±2	0.00

Table 2: Effect of *M. aeruginosa* extract on GOT, GPT and alkaline phosphatase in serum of rat

Days after treatment	ALP MI	GOT µl	GPT MI
1	154±9.3	63±4.1	70±1.8
2	169±10.4	77±8.2	88±4.2
3	179±8.07	71±2.5	95±3.6
4	182±9.1	123±6.2*	107±6
5	219±5.5*	108±5*	127±4.4*
Control	152±10.5	69±3.8	72±2.4

*Significant at $P < 0.05$

ALP: Alkaline phosphatase.

GOT: Glutamate oxaloacetate transaminase.

GPT: Glutamate pyruvate transaminase.

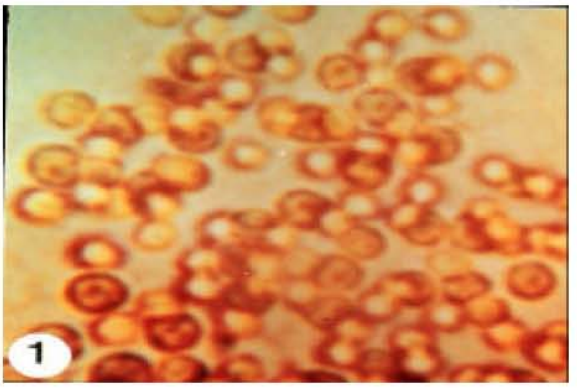

Fig. 1: Microscopic photography of the cyanobacterial cells X1500.

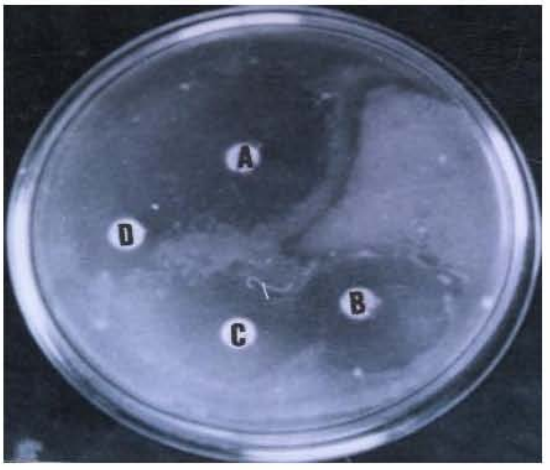

Fig. 2: Inhibitory effect of extract of *M. aeruginosa* against *Pseudomonas aeruginosa* at different dilutions (A) 1:10 w/v (B): 20 w/v © 1:30 w/v. D: 1:40 w/v.

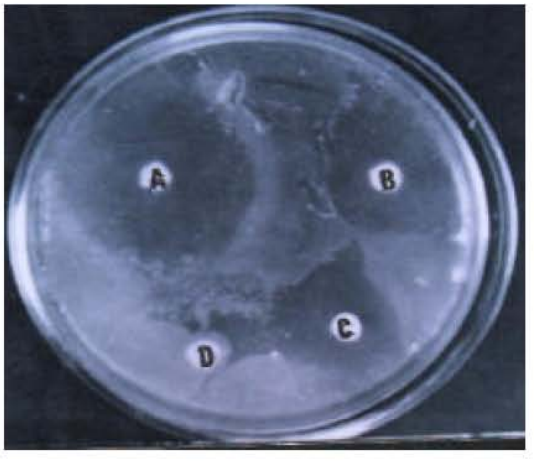

Fig. 3: Inhibitory effect of extract of *M. aeruginosa* against *Bacillus subtilis* at different dilutions (A) 1:10 w/v, (B) 1:20 w/v, © 1:30 w/v and (D) 1:40 w/v.

With regard to Gram -ve bacterium *P. aeruginosa* the inhibition zone diameter recorded a maximum of 34 ± 2mm at the first dilution 1 : 10 w/v. At the second dilution of the cell free extract in water 1 : 20 w/v the inhibition zones were of mean diameter of 24 ± 3 mm, while at the third dilution 1 : 30 w/v the mean diameter of the inhibition zones was about 19 ± 2 mm and the lowest diameter of inhibition zones was recorded around discs saturated by the cell free extract at the fourth dilution in water 1 : 40 w/v measuring 10 ± 3 mm diameters. In case of Gram +ve bacterium (*Bacillus subtilis*), the inhibition zone diameters were maximum at the first dilution 1 : 10 w/v with a mean value of 28 ± 4 mm, at the second dilution 1 : 20 w/v the mean diameter of inhibition zone was 18 ± 2 mm, while around discs saturated by cell free extract at third dilution 1 : 30 w/v, the mean value of inhibition zones diameter was 15 ± 2mm. The fourth dilution of the cell free extract in water 1 : 40 w/v was not effective I. e. no clear inhibition zones were observed around discs saturated by the extract on any of the growth plates seeded by *Bacillus subtilis*.

Hepatotoxic effect: Data in Table 2 summarized the results of the assayed enzymes in the blood serum after treatment with the cell free extract of *M. aeruginosa*. There was an elevation in the activity of alkaline phosphates in the serum of treated

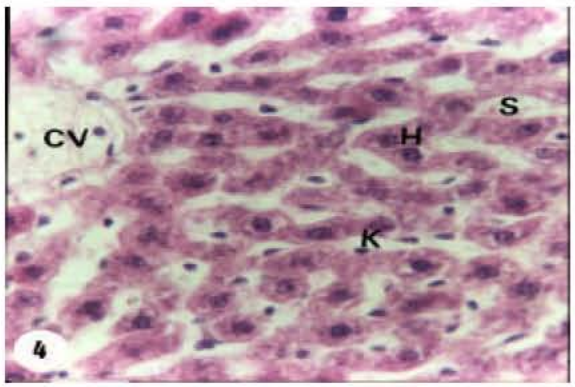

Fig. 4: Section in the liver of a control rat showing lobules formed of hepatocytes (H), CV: central vein, S: sinusoidal space contain Kupfer cells (K), X 300

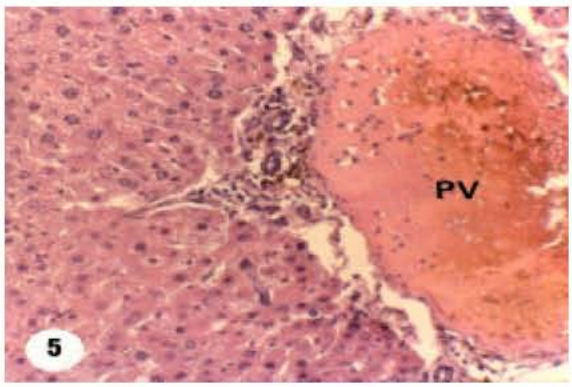

Fig. 5: Section in the liver of a rat after one day of treatment showing leucocytic infiltration (L) and activated Kupfer cell (arrow), X 300

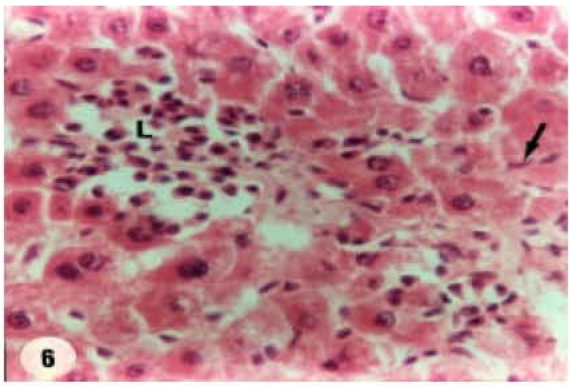

Fig. 6: Section in the liver of a treated rat showing dilated and congested portal vein (PV) X164

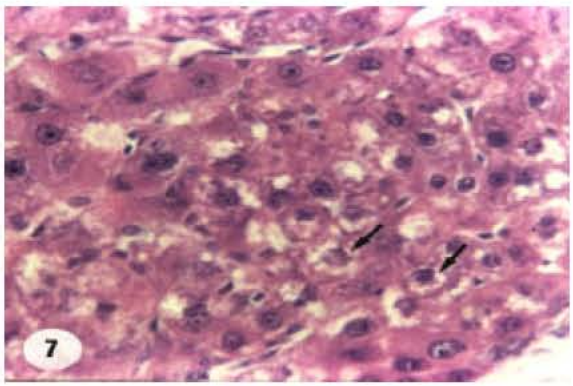

Fig. 7: Section in the liver of a rat after 5days of treatment showing cytoplasmic vacuolation of the hepatocytes (arrows), X300

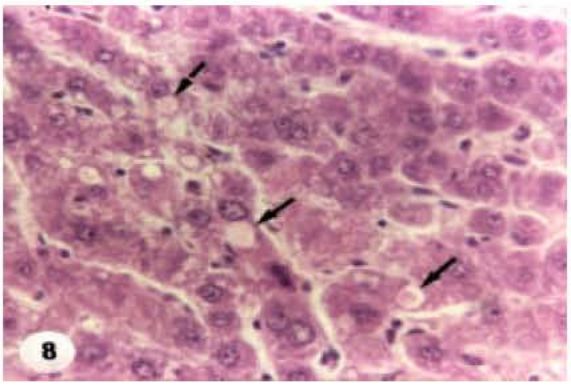

Fig. 8: Section in the liver of a treated rat showing fatty infiltrations (arrow), X 300

animals compared with that of control. The level of this enzyme increased significantly ($P>0.05$) after 5 days. Transaminases (GOT and GPT) also increased in the treated animals. The levels of GOT was significantly raised ($P>0.05$) after 4 and 5 days, while the level of GPT showed a

significant increase after 5 days.

Concerning the histological results, Fig. 4 showed that there was no histological changes observed in the liver of untreated (control) animals and examined at the same period as the treated ones. Examination of liver of animals after 1 and 3 days of treatment showed that the hepatocytes appeared with definite swelling. The sinusoidal spaces were found to contain activated kupffer cells and inflammatory leucocytic infiltrations were abundant (Fig. 5). The portal as well as central veins were dilated and congested (Fig. 6). Such congested blood vessels indicate clear phenomena of hemorrhage. Such histopathological changes existed and increased in animals examined after 5 days. Most of the hepatocytes manifested clear necrotic signs and others appeared with cytoplasmic vaculation (Fig. 7). Moreover, a large number of cells appeared to be suffering from fatty infiltrations (Fig. 8).

Discussion

The size of phytoplankton in water and its components were recorded by many authors to be important factor determining the features of the aquatic life specially in fresh water. Cyanobacteria as one of the abundant microbial groups throughout the phytoplankton represent about 17% of the phytoplankton (Shaaban *et al.*, 1999), autotrophically growing in fresh water ponds have many direct and indirect effects on aquatic life. It was observed by Shaaban *et al.* (1999 & 2000) that there are a certain correlation between the seasonal variation in cyanobacteria and both fish growth and bacterial counts. A certain cyanobacterium was found abundant forming condense blooms on the water surface, it was then important to characterize and identify that unknown bacterium.

Results obtained in present work revealed that according to *In vivo* growth, forming dense blooms in addition to *In vitro* colonial and microscopic studies and growth on the specific medium the microorganism was characterized and identified as *Microcystis aeruginosa* according to Prescott (1978).

Microcystis aeruginosa, is known for its toxicity and biological activities upon aquatic and terrestrial organisms (Watanabae *et al.*, 1981; Carbis *et al.*, 1995; Kim *et al.*, 1997 and Frazier *et al.*, 1998).

Cell free extracts showed potent antimicrobial activities against both *Bacillus subtilis* (Gram +ve) and *Pseudomonas aeruginosa* (Gram -ve) showing inhibition zones (clear zones or zones of no bacterial growth) which varied in diameter with the concentration of cell free extract diluted with water. The higher the concentration the larger the inhibition zone. This confirmed the seasonal correlation between counts of cyanobacteria and counts of true bacteria in water recorded earlier (Shaaban *et al.*, 1999 and 2000). The results may make it important to direct the attention towards the cyanobacterium to isolate and purify some antibacterial compounds effective specially against Gram –ve bacteria which are to some extent resistant to many of the known antibiotics. Biological activities of Cyanobacteria against many organisms are investigated and showed positive results against rotifers (Gilbert 1996), algae (Papke *et al.*, 1997) and fungi (Patterson & Bolis, 1997).

In regard to hepatotoxic effect, results revealed a significant increase in alkaline phosphatase and transaminase in sera of animals treated with *M. aeruginosa* extract. Similarly Nakano *et al.* (1989) found an elevation in GOT and GPT activities in sera of mice after the injection with the k-139 cells of *M. aeruginosa*. Carbis *et al.* (1994) reported an elevation in GIDH (glutamate dehydrogenase), γ-GT (Gamma-glutamyl transferase, aspartate aminotransferase (AST) and alkaline phosphatase in sheep exposed to *M. aeruginosa*. A highly significant increase in γ-GT was recorded in blood of patients obtaining drinking water from the supply contaminated with *M. aeruginosa* (Falconer *et al.*, 1983).

Results also showed that *M. aeruginosa* extract induced many histopathological changes in liver of rats. Similarly, Heaney (1971) demonstrated hepatic damage in mice treated with *M. aeruginosa* extract. The liver became dark red, had congestion in centrilobular areas, swollen cells and localized subcapsular hemorrhaging. Falconer *et al.* (1981) found that the administration of microcystins produced by *M. aeruginosa* to mice, at toxic levels induced bleeding and necrosis of the liver, and irreversible hypovolemic shock within 3 hours. Theiss *et al.* 1988, reported that in mouse and rat, microcystin-LR, produced by *M. aeruginosa*, is a potent rapid-acting and direct hepatotoxin with the immediate cause of death in acute toxicities being hemorrhagic shock secondary to massive hepatocellular necrosis.

It was reported that hepatocellular damage could be correlated with the disturbed enzymes activities. The elevation of serum alkaline phosphatase was considered as an indicator of early cholestatic liver damage and as a sensitive index in the early diagnosis of infiltrative diseases (Schiff and Schiff, 1982). Transaminases were considered to be more sensitive measure in evaluating liver function and damage (Sherlock, 1981). Martin *et al.* (1983) announced that liver tissues, which are known for their high content of transaminases (GOT, GPT) lost their enzymes in case of liver cell damage. This ultimately leads to their elevated levels in the sera of those animals. Treating rats with *M. aeruginosa* extract in the present work raised the levels of serum transaminases. This result together with the histological observations indicated that the used extract contains toxic substances (microcystins) (Botes *et al.*, 1982) responsible for the hepatic damage observed in the treated rats.

Acknowledgment

The authors would like to express their thanks to Mr. E. H. Sobhy, Institute of Oceanary and fishing Research, Egypt for his help in photography.

References

Allen, M.M., 1968. Simple conditions for growth of blue green algae on plates. J. Phycol., 4 : 1-4.

Botes, D.P., H. Kruger and C.C. Viljoen, 1982. Isolation and characterization of four toxins from the blue green alga. *M. aeruginosa*. Toxicon., 20 : 945-954.

Carbis, C. R., J.A. Simons, G.F. Mitchell, J.W. Anderson and I. McCauley, 1994. A biochemical profile for predicting the chronic exposure of sheep to *M. aeruginosa*, a hepatotoxic species of blue green algae. Res. Vet. Sci., 57 : 310-316.

Carbis, C. R.. D.L. Waldorn, G.F. Mitchell, J.W. Anderson and I. McCauley, 1995. Recovery of hepatic function and latent mortalities in sheep exposed to the blue green alga *M. aeruginosa*. Vet. Rec., 137: 12-15.

Charmichael, W.W., C.L. Jones, N.A. Mahmood and W.C. Theiss, 1985. Algal toxins and water-based diseases. CRC Critical Rev. Env. Cont., 15: 275-313.

Charmichael, W.W., 1988. Toxins of fresh water algae In: Handbook of Natural toxins., 3:121 New York, Mared Dekkar.

El-Sheekh, M.M. and A. A. Rady, 1995. Temperature shift-induced changes in the antioxidant enzyme system of cyanobacterium Synechocystis. Biologra Plantarum 37: 21-25.

Falconer, I.R., A.R.B. Jackson, J. Langley and M.T.C. Runnegar, 1981. Liver pathology in mice in poisoning by blue green algae *M. aeruginosa*. Aust. J. Biol. Sci., 34 : 179-187.

Falconer, I. R., A. M. Berespord and M.T.C. Runnegar, 1983. Evidence of liver damage by toxin from a bloom of the blue-green alga *M. aeruginosa*. Medical J. Aust., 1: 511-514.

Frazier, K., B. Colvin, E. Styer, G. Hullinger and R. Garcia, 1998. Microcystin toxicosis in cattle due to overgrowth of blue green algae. Vet. Human Toxicol., 40: 23-24.

Garg, S.K. and A. Bahatnagar, 1996. Effect of varying doses of organic and inorganic fertilizers on plankton production and fish biomass in brackish water fish ponds. Aquacult. Res., 27: 157-166.

Gilbert, J.J., 1996. Effect of food availability on the response of planktonic rotifers to a toxic strain of cyanobacterium *Anabaena flosaqua*. Limnol. Oceanogr., 41: 1565-1572.

Heaney, S.I., 1971. The toxicity of *M. aeruginosa* in pure culture. Phycol. News Bull., 8 : 5

Izzo, A.A., G. DiCarlo, D. Biscardi and R. De Fusco, 1995. Biological screening of Italian medicinal plants for antibacterial activity. Phytotherapy Res., 9: 281-286.

Kim, B.H., M.K. Choi, Y.T. Chung, J.B. Lee and I.S. Wui, 1997. Blue green alga *Microcystis aeruginosa* Kutz in natural medium. Bull. Environ. Contam. Toxicol., 59: 35-43.

Knud-Hansen, C.F. and T.R. Batterson, 1994. Effect of fertilization on the production of Nile Tilapia. Aquacult., 123: 271-280.

Martin, D.W., P.A. Mayes and V.W. Rodwell, 1983. Harper's review of Biochemistry. Middle East Edition, California.

Nakano, M., Y. Nakano, T. Saito-Taki, N. Mori, M. Kojima, A. Ohtake and M. Shirai, 1989. Toxicity of *Microcystis aeroginosa* K-139 strain. Microbiol. Immunol., 33 : 787-792.

Papke, U., E.M. Gross and W. Francke, 1997. Isolation, identification and determination of the absolute configuration of Fischerellin B, a new algicide from the fresh water cyanobacterium *Fischerella musicola*. Tetrahedron Lett., 38 : 379-382.

Parker, D.L., H.D. Kamar, L.C. Rai and J.B. Singh, 1997. Potassium salts inhibit growth of the cyanobacterium *Microcystis spp*. in pond water and defined media. Appl. Environ. Microbiol., 63: 2324-2329.

Patterson, G.M.L. and C.M. Bolis, 1997. Fungal cell wall polysaccharide elicit an antifungal 2^{ry} metabolite (phytoalexin) in the cyanobacterium *Scytonema ocellatona*. J. Phycol., 33: 54-60.

Prescott, G.W., 1978. How to know the fresh water algae. 3rd edition, University of Montana, pp: 235-238.

Schiff, L.A. and E. R. Schiff, 1982. Disease of liver, 5th ed. Philadelphia, Lippincott Company.

Shaaban, M.T., E.A. Khallaf, Z.A. Nagdy and M.A. El-Gammal, 1999. Variation of biological dynamics and physicochemical fluctuations in water of fish ponds, due to different (organic and inorganic) fertilizers applications. J. Union Arab. Biol., 8: 293-313.

Sendecor, G. and W. G. Cochran, 1971. Statistical methods, 6th ed. The Iowa State University Press, USA.

Shaaban, M.T., Z.A. Nagdy and M.A. El-Gammal, 2000. Population of certain bacterial groups in fertilized fish ponds. Proc. 1st Int. Conf. Biol. Sci. (ICBS) Fac. Sci., Tanta Univ. Egypt, 1: 637-651.

Sherlock, S., 1981. Disease of liver and biliary system, 8th ed. Oxford, Blackwell Scientific Publications.

Theiss, W.C., W.W. Charmicheal, J. Wyman and R. Bruner, 1988. Blood pressure and hepatocellular effects of the cyclin hepatopeptide toxin produced by the fresh water cyanobacterium, *Microcystis aeruginosa*. Toxicon., 26: 603-613.

Turell, M.J. and J.L. Middelbrook, 1988. Mosquito inoculation: An alternative bioassay for toxins. Toxicon., 26: 1089 – 1094.

Watanabe, M.F. S. Oishi and T. Nakao, 1981. Toxic characteristics of *M. aeruginosa* verh. Int. Verien. Limnol., 21: 1441-1443.

Watanabe, M.F. and S. Oishi, 1983. A highly toxic strain of the blue green alga *Microcystis aeruginosa* isolated from lake Suwa. Bull. Jpn. Soci. Fish, 49: 1759–1765.