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The Influence of *Lyngbya* Sp. and *Oscillatoria* Sp2 Isolated from Paddy Fields in Mazandaran Province, Iran by Bioassay

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Abstract: *Background and objectives*: Cyanobacterial blooms sometimes occur in large numbers of fresh and brackish water sources worldwide. A number of different species produce toxins of several different types. The most common toxins fall into two main classes: neurotoxins and hepatotoxins. To investigate their toxic effects, strains were isolated from paddy fields in Mazandaran Province, Iran during the spring. *Materials and Methods*: Mouse and *Daphnia magna* bioassays were performed on samples during months in which cyanobacteria dominated. Cyanobacteria were grown separately for 20 days in their specific culture mediums before experimental use in the culture room at 27±2°C under 75 µmol $m^{-2}s^{-1}$ photon flux compactness with a photoperiod of 14 h light: 10 h darkness. The isolated Cyanobacteria were exposed to a combination of yellow and white beams of light. *Results*: Three of genera of *Lyngbya* sp and *Oscillatorias* sp2 were identified as toxic algae and LD_{50} values by intraperitoneal injection were 250mg/mL and 525mg/kg, respectively. A bioassay using *Daphnia* revealed LC_{50} values of 0.2 mg/mL and 0.14 mg/mL, respectively. Signs and symptoms of intoxication caused by *Oscillatoria* sp2 in a mouse showed hepatotoxic and neurotoxin characteristics. This toxin was a cyclic microcystin. Signs and symptoms of intoxication caused by *Lyngbya* sp in a mouse model showed neurotoxin characteristics. This toxin was an alkaloid saxitoxin. *Conclusion*: The intensity and importance of the damage and injuries to humans, mammals and aquatic biota caused by cyanobacteria demand that the quality and quantity of the water sources should be highly valued.

Key words: *Lyngbya* · *Oscillatoria* · Toxicity · Biological assay

INTRODUCTION

Cyanobacteria provide an extraordinarily wide-ranging contribution to everyday life and are of economic importance [1]. Recently, several investigators have noted that in China, Africa, North and South America and Australia, people will use water from sources with green algae by digging holes (soaks) near the water's edge in order to filter the water through the ground and thus prevent the green material from contaminating drinking-water supplies. Cyanotoxins belong to diverse groups of chemical substances, each of which shows specific toxic mechanisms in vertebrates. Some cyanotoxins are strong neurotoxins (anatoxin-a, anatoxin-a(s), saxitoxins), while others are primarily toxic to the liver (microcystins, nodularin and cylindrospermopsin) and yet others (such as the lipopolysaccharides) appear to cause health impairments (such as gastroenteritis), which are poorly understood. Microcystins are geographically widely distributed in bodies of freshwater, but recently, they have also been identified in marine environments as agents of liver disease in net-pen reared salmon, although it is not clear which organism in marine environments contains these toxins [2].

Light and electron microscopy has revealed that cyanobacteria morphology comprises unicellular, colonial and multicellular filamentous forms. In contrast to eukaryotic microalgae, cyanobacteria do not possess membrane-bound subcellular organelles; they have no discrete membrane-bound nucleus; they possess a wall structure based upon a peptidoglycan layer; and they contain 70 S rather than 80 S ribosomes [3]. Cyanobacterial blooms in freshwater usually comprise both toxin- and non-toxin-producing species.

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The main toxin-producing cyanobacteria genera include *Anabaena*, *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Lyngbya* and *Cylindrospermopsis* [4]. Cyanotoxins (hepatotoxins and neurotoxins) are responsible for intoxication of wild and domestic animals, contamination of drinking water, inducing fish mortality and the elimination of aquatic biota [5,6]. Microcystins are produced by four planktonic cyanobacteria genera: three are filamentous (*Anabaena, Oscillatoria* and *Nostoc*), while one is coccoid (*Microcystis*). *Microcystis* produces the greatest variety of toxins by incorporating a variety of different L-amino acids in the microcystin molecule [7-9].

In mouse bioassays, which traditionally have been used to screen toxicity of field and laboratory samples, cyanobacterial hepatotoxins cause death by liver hemorrhage within a few hours of acute dose administration [4].

The crustacean water flea (Daphnia magna) is a well-known standard test species in freshwater ecotoxicological studies and detailed protocols are available for its use in both ecological risk assessment and reformative tests with chemicals [10]. *D. magna* is widely used due to its small size, easy handling and laboratory culture, wide distribution, availability throughout the year, predominant parthenogenesis reproduction, ecological relevance in food chains and high sensitivity [11,12]. Bioassays are convenient initial tests to estimate the toxicity of waterbloom material and laboratory cultures or cell extracts [13].

The aim of the present study was to investigate the effects Cyanotoxins in fresh water ecosystems of Damavand City, Iran. Herein, we showed that cyanobacterial *Oscillatoria* sp. was toxic to *D. magna* and to mice.

MATERIALS AND METHODS

Sampling: The sampling period for the present survey was from September through December 2010. Water samples for the bioassay were collected from irrigated paddy fields in Mazandaran Province, Iran. The 3 L samples were collected in sterile polythene bags in duplicates from a depth of ~30 cm depth and 1 m from vegetation and immediately transported to our laboratory.

Cultivation and Isolation of Cyanobacteria: Cells were harvested by centrifugation and stored at -20°C until they were analyzed. Prior to harvesting, each culture was examined microscopically. The cultures that showed evidence of contamination were homogenized in a blender, washed three times in BG-11 (American Type Culture Collection (ATCC) 616) medium using low-speed centrifugation (500 x g) for 20 min and separated on a 10-90% Percoll gradient (Sigma_Aldrich, St. Louis, MO, USA) at 2500 x g or at higher speeds, depending on the amount of mucilaginous material present in the preparation. Bands containing cyanobacteria were harvested, washed two times in medium and examined microscopically for purity [7].

Strains were grown in artificial seawater nutrient (ASN)-III culture medium with or without combined nitrogen supplementation depending upon whether the cyanobacteria were heterocystous of non-heterocystous [16]. Cyanobacteria were grown for 20 days in their respective growth media before experimental use and maintained in the culture room at 27±2°C under 75 μmol $m^{-2}s^{-1}$ photon flux density(PFD) at 50 microeinsteins (pH =7) with a photoperiod of 14 h of light:10 h of darkness while being rotated.

Toxicity bioassay tests. *D. magna* and mice (*Mus musculus*) were subjected to acute toxicity testing of algal extracts in the present study.

Preparation of Crude Cyanobacterial Extract: Lyophilized bloom material was dissolved in distilled water, frozen and thawed at room temperature. This freeze/thaw cycle was repeated four times. After the last cycle, the thawed material was ultrasonicated for 10 min [17, 18] and then particulate matter was removed by centrifuging at 3500 g for 30 min.

D. magna. A freshwater *D. magna* strain that was successfully grown in the laboratory in synthetic freshwater media (SFM) was used in the present study. Twenty neonates (< 24 h old) were transferred to 250 mL glass beakers containing 100 mL of SFM and placed in a 70 x 60 x 30 cm aquarium. As a control, un-inoculated SFM was used. A mercury thermometer was used to measure temperature in the test containers, which was maintained at 22± 2ºC. Acute toxicity testing was conducted in triplicate, where groups of 10 < 24 h-old daphnias were placed in 250 mL beakers containing 100 mL of medium and subjected to test conditions for 24 h. Tests were run without food addition. A control test was run in parallel. Treatments with different concentrations of wet weight (in mg/mL: 0/2, 0/4, 0/8, 1/1, 2/1, 2/5, 3/1, 5/1, 10/1 and 20/1) of *Oscillatoria* were performed three or more times to determine the LC_{50} values of the *D. magna* specimens [19, 20].

Mice: The toxicity of freeze-dried algal material was determined using twelve male Swiss mice weighing 20-25 g (three mice per treatment). Toxicity was tested by intraperitoneal (i.p.) injection of algal cell lysate dissolved in 0.9% NaCl at a concentration of 100, 400, 700 and 2000 mg (wet weight)/mL and suspended by vortexing for 30 min followed by 30 min of centrifugation at 4000 rpm [21]. Mice were i.p. injected with 1 mL of algal cell lysate solution, whereas control mice were injected with 1 ml of 0.9% NaCl solution. The injected animals were observed for up to 24 h for any signs of poisoning. Toxicity was quantified as LD_{50} (the lethal dose for 50% of tested animals) [22].

RESULTS

Two genera of cyanobacteria, *Oscillatoria* and *Anabaenasp*, were identified by microscopy. An acute toxicity bioassay was performed by exposing *D. magna* (neonates, 24 h old) to water extract of the dominate blue-green algae (*Oscillatoria sp* or *Lyngbya sp*.) collected from paddy fields in Mazandaran Providence. Tables 1 and 2 show the percentages of mortality of *D. magna* observed after 24 h exposure to various concentrations of crude cyanobacterial extract.

Table 1: Effect of Blue-Green *Oscillatoria sp 2* on *Daphnia magna*

concentrations Control (mg/ml)	Initial Neonates Number	Death Neonates After 24h
0/2	20	0
0/4	20	1
0/8	20	5
2/1	20	8
2	20	11
2/5	20	11
3	20	17
5	20	19
10	20	20
20	20	20

Table 2: Effect of Blue-Green *lyngbya sp* on *Daphni a magna*

Contro l concentrations (mg/ml)	Initial Neonates Number	Death Neonates After 24h
0/2	20	0
0/4	20	2
0/8	20	6
2/1	20	11
2	20	16
2/5	20	16
3	20	18
5	20	20
10	20	20
20	20	20
0/2	20	0

Table 3: Symptom and multiplicity mortality of blue-green *Oscillatoria sp2* and *Lyngbya sp.* extracts on mice

Concentrations cyanobacteria (mg/kg)	100 mg/kg	400 mg/kg	700 mg/kg	1000 mg/kg	(%)
Oscillatoria sp2	Inactive, Stolid	Inactive, Blind, Bloodshed ear 1dead	4 dead	4 dead	0/09-0/17
Lyngbya sp	Inactive, Stolid	Inactive, Gasp, Blind, 3 dead	4 dead	4 dead	0/06-0/07
Control (0.9% NaCl only)	NE	NE	NE	NE	0/06-0/07

Table 4: Toxicity of blue-green *Oscillatoria sp 2*. extracts on mice

Concentrations (mg/kg)	Neurotoxin (1-30 min)	Hepatotoxin (45-240 min)	Hepato+Neuro Toxic (15-30 min)
Control (0.9% NaCl only)	NE	NE	NE
100	NE	NE	NE
400	NE	NE	1 dead
700	- NE	NE	4 dead
1000	- NE	NE	4 dead

Table 5: Toxicity of blue-green *Oscillatoria sp 2*. extracts on mice

Concentrations (mg/kg)	Neurotoxin (1-30 min)	Hepatotoxin (45-240 min)	Hepato+Neuro Toxic (15-30 min)
Control (0.9% NaCl only)	NE	NE	NE
100	NE	NE	NE
400	3 dead	NE	NE
700	4 dead	NE	NE
1000	4 dead	NE	NE

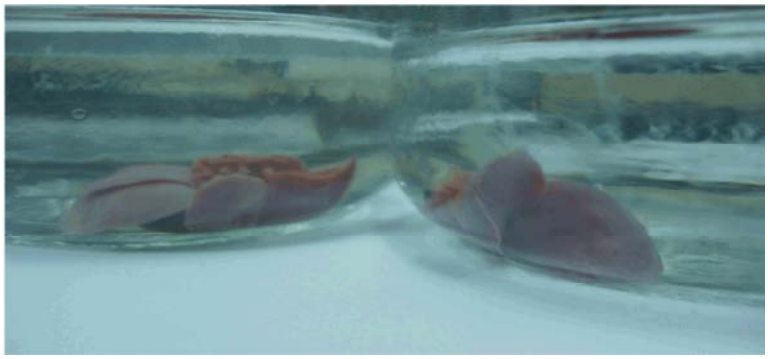
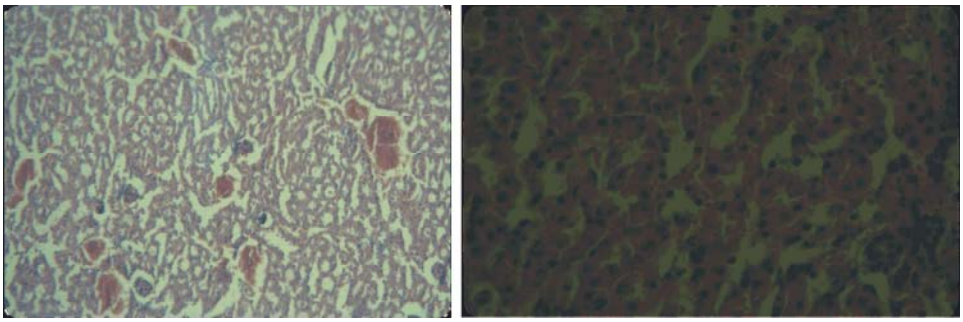
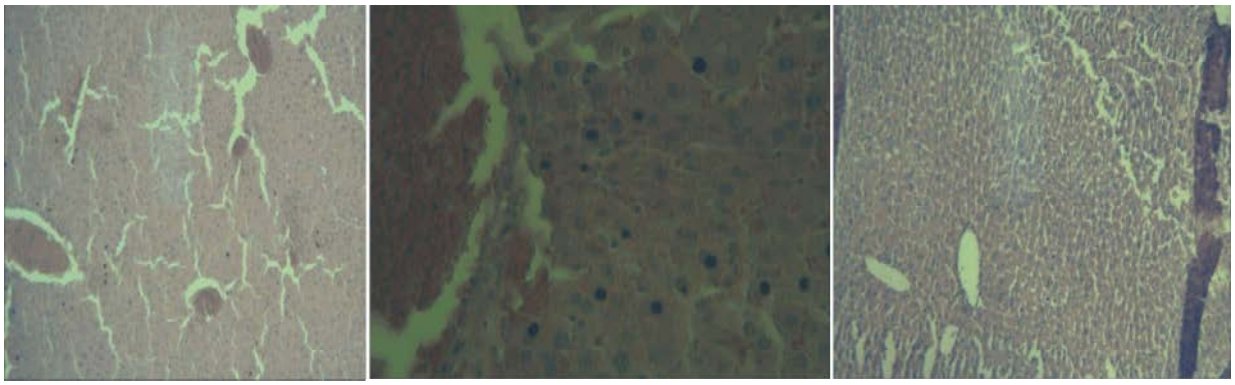


Fig. 1: Morphological changes in mouse livers. Left: Control Right: treated



(a) (b)

Fig. 2: a. *Oscillatoria* sp. b. *Lyngbya* sp



a1 a2 b

Fig. 3: Bloodshed. (b) kidney tissue in control mouse *Oscillatoria* sp2a1-a2- bloodshed and necrosis (b) Liver tissue in control mouse. Arrows indicate hyperchromatin that can result in necrosis.

Dissolved oxygen content during the bioassays varied between 5.1 and 7.5 mg/L and the lowest oxygen concentration was observed in the highest concentrations of cyanobacterial extracts. The pH values increased slightly at the end of the experiments and did not change more than 0.3 units (range, 6.5 to 7.7) at most concentrations tested (data not shown).

Four mice were used to test the toxicity of each *Oscillatoria* and *Anabaena sp.* The injected animals were observed up to 24 hrs. Behavioral symptoms and survival times are shown in Table 3. The results showed that injection of *Anabaena sp.* extract into mice did not yield any signs of neurotoxicity or hepatotoxicity (Table 3). Toxicity of blue-green *Oscillatoria* sp2 and *Lyngbya* sp to mice is shown in Tables 4 and 5. This toxin was identified as a cyclic microcystin.

Mice injected with *Oscillatoria* sp showed signs of neurotoxicity and hepatotoxicity, although postmortem examinations failed to show changes in gross morphology or in any organ including the liver and kidney (Figs 1 and 2). Mice injected with *Lyngbya sp*. extract did not yield any signs of hepatotoxicity.

Acute toxicity tests showed that the endocellular toxins of *Oscillatoria sp and Lyngbya sp.* were toxic to *Daphnia* and mice.

DISCUSSION

Toxins produced by cyanobacteria have been reported in marine and freshwater environments worldwide. Toxigenic cyanobacteria are common components of surface waters in Florida and may pose a threat to ecosystems and human health. Data (n = 72 for all toxin analyses) showed that microcystins (0.1-3.6 µg/L) were present year-round and multiple bloom events in each lake were observed. Bloom concentrations ranged from 5-7,500 µg/L and blooms occurred throughout the year [23].

Results of this survey showed that extracts from *Oscillatoria sp and Lyngbya sp.,* which contain microcystins, was toxic to *Daphnia* and mice. In contrast, Nizan *et al*. [24] have demonstrated that the toxicity of *Microcystis* strains to mice does not always coincide with the toxicity to *Daphnia.* Jungmann and Benndorf [25] found that extracts containing *Microcystis aeruginosa,* which did not contain microcystins, was much more toxic to *Daphnia* than another fraction containing microcystins. Also, Pereira *et al*. [26] obtained negative results for cyanobacterial toxicity during a *Planktothrix mougeotii* bloom using a mouse bioassay. *M. aeruginosa* were found to have different short-term toxic effects on *D. magna*, as *M. aeruginosa* 7820 was found to be toxic in the mouse assay by inhibiting food uptake. Despite the small amounts consumed, the toxicity was sufficient to induce rapid mortality of *D. magna* [21]. Lampert [27] showed that aged cultures of cyanobacteria are more toxic than fresh cultures. The LC_{50} of microcystins for *Daphnia* at 48 h was 3.15 g/L and the LC_{50} of microcystin at 24 was 3~17 µg/mL [21, 27]. In the present study the LC_{50} values of cyanobacteria were in agreement with previous studies as the relationship between injected dose and LT_{100} (lethal time it takes a pathogen to kill 100% of the infected individuals) was similar. In the present study, the relationship between microcystin concentration and lethal dose (LD) time response in the *D. magna* bioassay was similar to the LD time response of the mouse bioassay. Here, the *D. magna* and mouse bioassays used i.p. injection of the toxic algal extract. Also, the relationship between cyanobacteria concentration and death time in the *Daphnia magna* bioassay was similar to the dose-lethal time reaction of the mouse bioassay. The relative toxicity of each cyanobacterial strain assessed using the *D. magna* bioassay was almost the same as that of the mouse bioassay. The results of the current mouse bioassay were in agreement with the results reported by Vezie *et al*. [28]. The sensitivity and time requirements of the *D. magna* bioassay were also compared with those of the mouse bioassay in this report. Many publications have shown that most microcystins display an LD_{50} value of 1~2 µg/mouse within 4 h [29]. When compared to the quantity of toxin used in the *D. magna* bioassay with the above LD_{50} value in mice, it seems that the sensitivities were less. However, the mouse bioassay will be less sensitive than the *D. magna* at observation intervals < 24 h. A mouse bioassay with field-collected *microcystis* showed a toxicity close to that obtained by Azevedo *et al*. (LD_{100} = 31 mg/kg mouse, i.p.) [30]. When compared to the quantity of toxin used in the present study on mouse bioassay will be less sensitive than it. Our data revealed that a minimum of 40 min was needed to observe the death effect in the mouse bioassay after i.p. injection of algal extracts at doses higher than the threshold. It has been reported that the time required for maximal toxin accumulation in the liver varied from 1 min [31] to 60 min [32] after microcystin-LR (MCLR), a cyclic heptapeptide produced by the blue-green algae *M. aeruginosa*, administration in mice. Signs and symptoms of intoxication caused by cyanobacteria *Oscillatoria sp2* in a mouse model showed heptotoxic and neurotoxic characteristics. Signs and symptoms of intoxication by *Lyngbya sp.* in a mouse model showed neurotoxin characteristics. This toxin was identified as an alkaloid saxitoxin. The intensity and importance of the damage and injuries to humans, mammals and aquatic biota caused by cyanobacteria demand that the quality and quantity of the water sources should be highly valued.

CONCLUSION

In this experiment, we show that although the *D.magna* bioassay is less sensitive and more time-consuming than the mouse bioassay, nevertheless, it is less constraining than the mouse bioassay because it requires less laboratory equipment and is less problematical from an ethical point of view. Thus, we suggest that mouse bioassay can be a convenient method for screening hepatotoxic cyanobacterial strains.

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