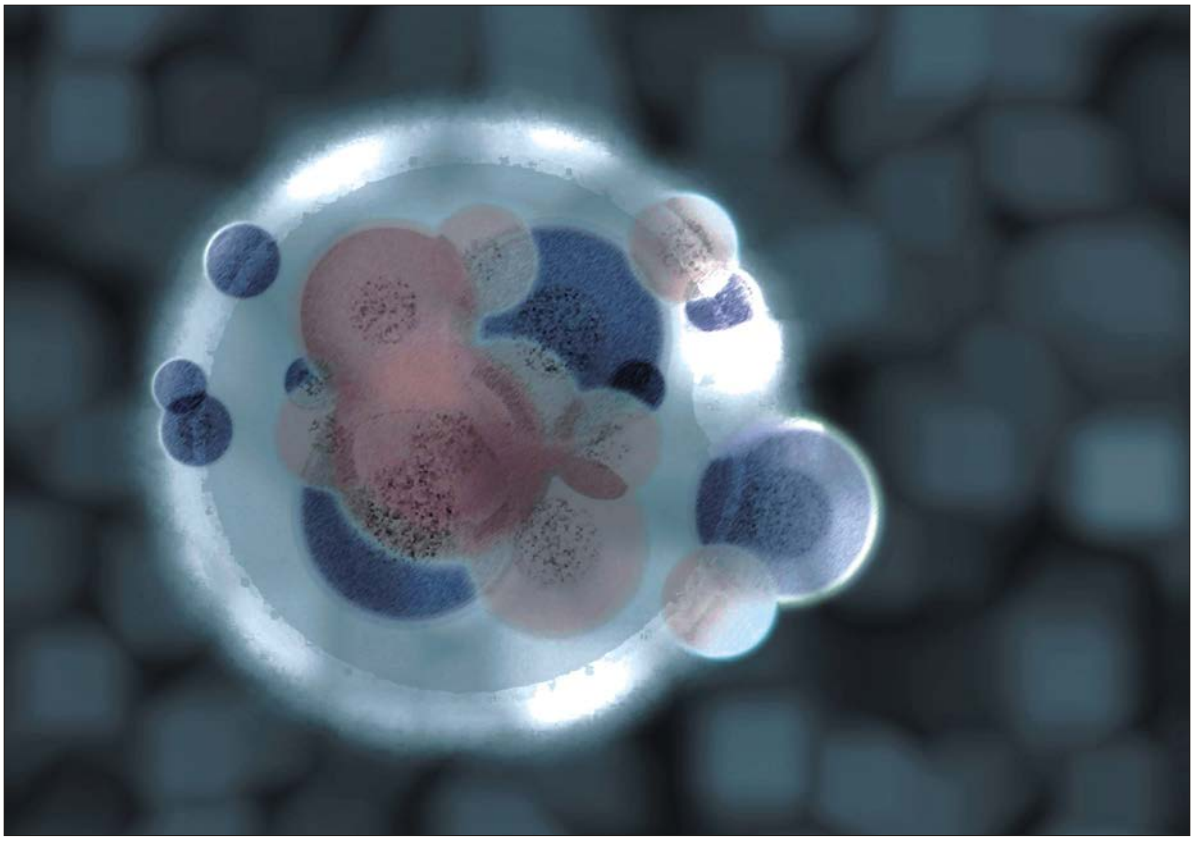

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First Report on the Identification of Nodularin from King Talal Reservoir (Jordan)

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Abstract: The aim of present study was to investigate *Nodularia spumigena* blooms intensity and their toxin concentration and biochemical characters in King Talal Reservoir. The time of the seasonal maximum intensity of this type of cyanobacterial blooms vary significantly from month to month during 2003. The maximum intensity of the bloom was from 30th of July-30th of Aug. The LD_{50} of nodularin in Balb/C mice was 44 μg kg^{-1} body weight (b.w.) and the maximum concentrations of nodularin in King Talal Reservoir water were 0.455 μg L^{-1} on 30th of July, 0.46 μg L^{-1} on 1st Aug and 0.45 μg L^{-1} on 30th of Aug. Therefore, it is considered as a potent biotoxin. By using NMR, it has shown that the structure of this peptide is similar to that found by others in other places of the world and biochemical studies on this toxin showed that more than 50% inhibition of PP1 occurred at these concentrations.

Key words: *Nodularia*, nodularin, King Talal Reservoir

INTRODUCTION

Cyanobacterial toxins are toxins produced by cyanobacteria, or blue-green algae. They include neurotoxins (e.g. annatoxins), hepatotoxins (e.g. microcystins), skin irritants and other toxins. Both hepatotoxins and neurotoxins are produced by cyanobacteria commonly found in surface water supplies and therefore appear to be of most relevance to water supplies at present[1]. However, the neurotoxins are relatively unstable and as such, are not considered to be as wide spread as hepatotoxins in water supplies.

Cyanobacterial species are widely recognized as a source of unpleasant odor in water supplies[2] due to production of some organic compounds with dissimilar biological activities including peptides, macrolids and glycosides[3]. These compounds have been reported to possess a number of bioactivities, antiviral[4] antifungal[3] cytotoxic[5] and antineoplastic activities[6]. Many of these compounds are toxic to animals and human. Incidence of human illness have been linked to the recreational use of water contaminated by cyanobacterial blooms, including *Microcystin, Nodularia*, *Oscillatoria* and *Anabaena* in certain area of the world[7]. These toxins are a very diverse group from both the chemical and toxicological standpoints. The substantial input of nitrogen and phosphorus compounds from municipal, industrial and agriculture wastes promotes the growth of phytoplanktonic organisms, which are the most frequently used indicators of eutrophication.

In King Talal Reservoir in Jordan (which represents the largest water basine in Jordan, located about 30 km north of Amman with geographical co-ordinates of 32°11 N and 35°48 E), the summer blooms of the cyanobacteria *Nodulatia spumigena* is of an annual occurrence. The growth is favoured by a low N:P ratio, calm weather and high solar radiation; the latter two factors are conductive to the formation of the thermocline.

The presence of *Nodularia spumigena* in King Talal Reservoir and possibly recreational water is of particular concern as it produces nodularin, a cyclic pentapeptide with hepatotoxic activity. The molecule of this toxin contains dehydroamino acid, *N*-methyldehydrobutyrine (Mdhb), two D-amino acids, D-glutamic (D-Glu) and D-erythro-*B*-methylasparic acid (D-MeAsp) and the more common L-arginine (L-Arg). Also, it contains the fatty acid C_{20} amino acid, *Nodularia* was the first cyanobacterium recognized to cause death for animals and human[8].

Nodularin is chemically stable[9,10] and if not diluted, can persist in water for several days or weeks after the bloom[11]. The hydrophobic Adda side chain and the cyclic structure of nodularin are essential for the molecule s biological activity[12]. At the cellular level, the toxin inhibits the activity of protein phosphatases (PP), which are key regulatory enzymes[13]. In liver, the inhibition of PP1 and 2A leads to cellular disruption and promotes tumour formation[14]. Most of the data on nodularin toxicity have been obtained from experiments on rodents[15]. Nodularin has proved to be not only a tumour promoter but a tumour initiator as well[16].

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Nodularin is synthesized and retained in *Nodularia* cells, but during bloom senescence cells are lysed and the toxin is released into the surrounding water. The aim of this study was to identify the species of *Nodularia* present in King Talal Reservoir, the exact period of their nourishment in this water basine, their toxin (nodularin) structure and concentration in the water and to determine the biochemical activity of this biotoxin.

MATERIALS AND METHODS

Chemicals: All chemicals used in this study were of analytical grade and purchased from Sigma Co. unless otherwise indicated.

Sampling: In the summer months of 2003 (June-Nov), cyanobacteria samples were collected twice a month from chosen sites of King Talal Reservoir. Surface water samples with suspended organisms were collected using one liter Nansen water sampler (Nansen Co; Germany). On the same day, the samples were passed through a Whatman GF/F glass microfiber filter. *Nodularia* cells were identified using light microscope according to the methods recommended previously[3,15,17]. The isolated cells were cultured using BG-11 medium according to Lehtimaki[3].

Extraction and analysis: To determine the total concentrations of cell bound and extracellular nodularin, a known volume of a water sample was sonicated for 5 min and passed through glass microfiber filter (Whatman GF/C). The filtrate was concentrated by solid phase extraction (SPE) using the method recommended by Mazur and Plinski[15].

For analysis of cell-bound nodularin, the method recommended by Paczuska and Kosakowska[18] was used with the following modification: Lyophilized cyanobacterial cells (50 mg) were placed in microcentrifuge tubes, 1 mL of 90% methanol was added to the samples, which were sonicated for 30 sec. After 1 h, the samples were centrifuged at 20,000xg for 20 min, supernatants were collected and filtered through 0.22 μm filter and applied directly to preconditioned C18 cartridge. The mobile phase was a mixture of 10% aqueous acetonitrile: 100% acetonitrile (60:40), both containing 0.05% trifluoroacetic acid (TEA), at the flow rate of mL/min as recommended by Lee *et al.*[19].

Nodularin was identified by its retention time and characteristic absorption spectrum with a maximum at 238 nm[15].

Mouse bioassay: Five to seven weeks-old male albino Balb/C mice (about 30 g wt. each) were used. Two groups of mice (six each) were injected i.p. with 1 mL of normal saline (control) or with 1 mL of 30 mg mL^{-1} (toxin treated).

Protein-phosphatase 1 assay: PP 1 activity was determined by measuring the rate of color production from the dephosphorylation of *para*-nitro-phenolphosphate (*p*NPP) substrate as a function of time using the microtiter plate reader according to the method recommended by An and Carmichael[20], Mackintosh (personal communication, 2003). 2-D-electrophoresis (Apelex; France) was used for assessment of the purity of the enzyme.

RESULTS AND DISCUSSION

The presence of *Nodularia* blooms in King Tala Reservoir was observed by us for the first time. In the present study, it was found that the time of seasonal maximum and intensity of *Nodularia* blooms in King Talal Reservoir vary significantly from month to month of the year (June-Nov.). Interestingly, a marked difference in the bloom intensity and dynamics between those months was observed. Using a method recommended[15], it was clear that the highest intensity of those blooms was during the period 30th of July-30th of August, 2003 (Fig. 1). *Nodularia* collected from King Talal Reservoir was identified as *Nodularia spumigna* (Whitton, 2003 personal communication; Sakar, 2003 personal communication; Carmichael, 2003 personal communication Lehtimaki[3]).

The summer bloom of *Nodularia spumigena* is favoured by a low N:P ratio, calm weather and high solar

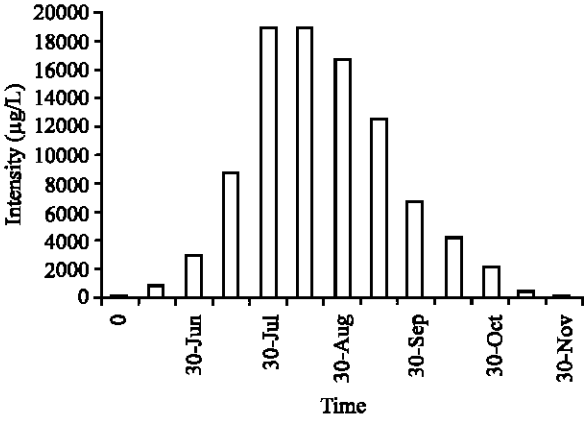

Fig. 1: *Nodularia spumigena* intensity in sample collected in the summer time of 2003 from King Talal Reservoir

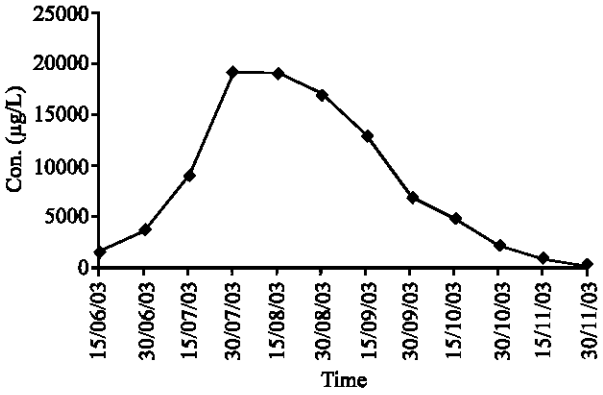

Fig. 2: Nodularin concentration in samples collected in the summer time from King Talal Reservoir

radiation; the latter two factors are conductive to the formation of the thermocline. In samples collected during this period (June-Nov.), the concentration of *Nodularia spumigena* toxin; nodularin was determined, its concentrations through this period (Fig. 2). During these intensive blooms of *Nodularia spumigena*, it was found that the cell-bound nodularin made up about 80-89% of the total toxin concentration.

The LD_{50} of nodularin is approximately 44 µg kg^{-1} b.w. in mice when administered intraperitonealy. With such low LD_{50}, nodularin of King Talal Reservoir is considered a potent natural toxin and seems to be stronger than that found by others[3] in Baltic sea[15] in Gdansk Gulf. According to Falconer[21] as well as Mazur and Pliniski[15] nodularin at such toxicity poses a high risk of adverse health effects if water is used for drinking.

Nodularin was identified by its retention time and characteristic absorption spectrum with maximum at 238 nm (Fig. 3) which is due to the conjugated diene in the structure of the unusual amino acid Adda[15]. In the lyophilised *N. spumigna* cells, the nodularin concentration varied between 2855-4420 µg g^{-1} d.w.

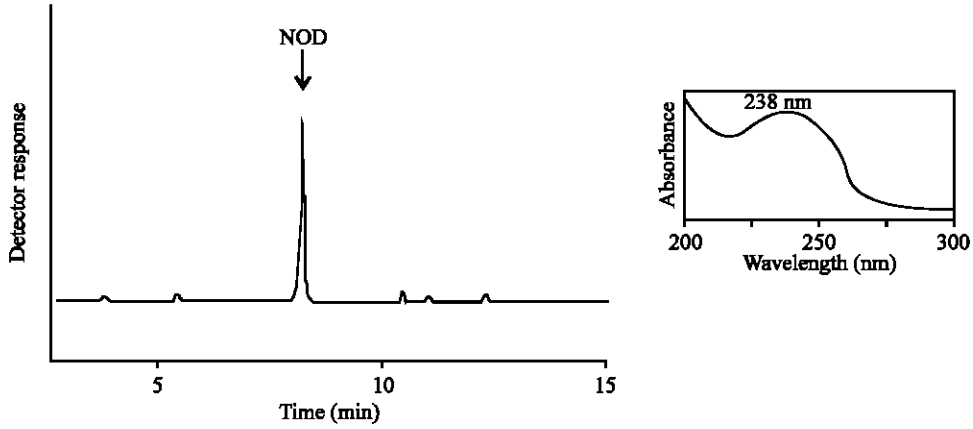

Fig. 3: HPLC chromatogram of *Nodularia spumigena* sample from King Talal Reservoir. The absorption spectrum of authenic nodularin

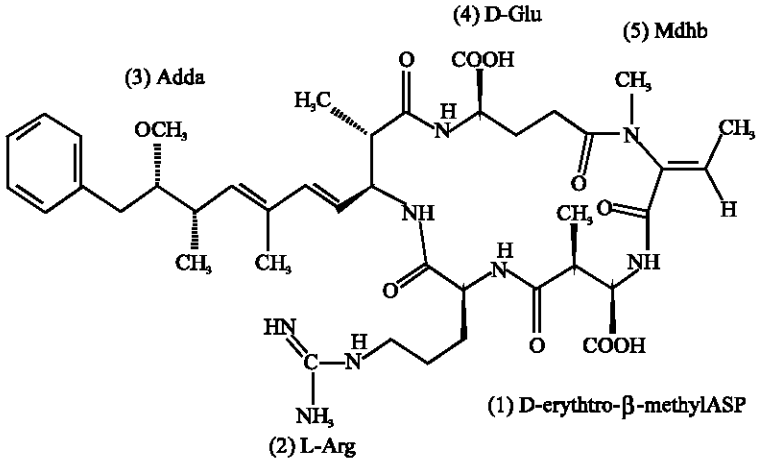

Fig. 4: The chemical structure of nodularin (NOD) of *Nodularia spumigena* sample from King Talal Reservoir

Quantitative analysis of the toxin was carried out using a calibration curve based on peak area measurements for the standard solution. In a single step extraction of lyophilised cells with 90% methanol, it was confirmed that almost the same amount of toxin (1.33 μg g^{-1} d.w.) was recovered as in two steps (1.31 μg g^{-1} d.w.) or three steps (1.34 μg g^{-1} d.w.) extractions.

The three-dimentional solution structure of nodularin was studied by NMR (Bruker; France) according to Annila method[22] and molecular dynamics simulations. The conformation in water was determined from the distance and dihedral data by distance geometry and refined by iterative relaxation matrix analysis. The cyclic backbone adopts a well defined conformation but the remote parts of the side chains of arginine as well as the amino acid derivative Adda (Fig. 4) have a large spatial dispersion. For the unusual amino acids, the spartial charges were calculated and nodularin was subjected to molecular dynamic simulations in water. The three-dimentional structure resembles very closely that of microcystin-LR[22] in the chemically equivalent segment. Therefore, it is expected that the binding of both microcystins and nodularins to serine/threonine-specific protein phosphatases is similar on an atomic level.

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