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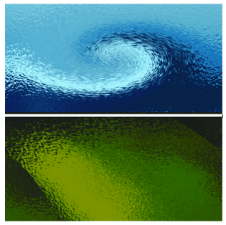

RESEARCH ARTICLE

First Observation of Microcystins in Tunisian inland waters: a threat to river mouths and lagoon ecosystems

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Abstract

1 - Microcystin pollution is known to affect different types of inland water bodies: river mouths and coastal lagoons may be affected by local production as well as by transportation through the freshwater network. Physicochemical and biological water quality, including the total microcystin concentration, was investigated from July to December 2003 in the reservoir Hjar, Tunisia.

2 - Microcystin levels and characterization of the different microcystin variants present were measured by protein phosphatase inhibition assays (PP2A) and by high performance liquid chromatography coupled to diode array detector and tandem mass spectrometry, respectively. The microscopic examination of the phytoplankton samples showed the dominance of the *Oscillatoria* and *Pseudoanabaena* genera. The total (particulate and dissolved) microcystin concentrations in the reservoir water varied between 23.4 and 7455.2 ng/l microcystin-LR equivalent per liter.

3 - The highest MCYST concentration was observed in November 2003. The analysis of the field bloom extract from this month by HPLC coupled to photodiode-array detector revealed the presence of five peaks having characteristic spectra of microcystins with a maximum of absorbance at 238 nm. HPLC/MS/MS analysis of this sample demonstrated the presence of three variants of microcystins: microcystin-LR (MCYST-LR), microcystin- (MCYST-RR), microcystin- (MCYST-YR). Therefore, estuaries of rivers contaminated by cyanobacteria toxins may play an important role on the transfer of these cyanotoxins through food chains.

Keywords: Cyanobacteria, Oscillatoria, Pseudoanabaena, Microcystin, Hjar reservoir.

Introduction

Cyanobacteria (blue-green algae) are ancient, cosmopolitan inhabitants of fresh-, brackish-, and marine waters, and terrestrial environments (Whitton and Potts, 2000). Therefore, cyanobacteria toxin poisonings (CTPs) occur in fresh (lakes, ponds, rivers and reservoirs), marine and transitional (river mouths and lagoon ecosystems) waters throughout the world (for an overview, see Sivonen & Jones, 1999). In transitional water ecosystems, cyanobacteria toxin poisoning can be produced locally as well as transported through the river networks. Being the transitional waters localized at the interface among terrestrial, freshwater and marine ecosystems, they are sink water bodies of nutrient and organic matter particularly exposed to the pollution.

The increasing number of cyanobacteria-infested water reservoirs used for drinking-, irrigation-, and recreation-water constitute a potential risk of public health for many populations. Several species are able to produce potent toxins that cause acute mortality in animals (Turner *et al.*, 1990; Carmichael &

Falconer, 1993) and illness in humans (Kuiper-Goodman *et al*., 1999) or, when exposed through hemodialysis even death (Jochimsen *et al*., 1998, Pourria *et al*., 1998). These health hazards have led the World Health Organization (WHO) to establish a provisional guideline value for microcystin-LR of 1 µg L-1 drinking water (WHO 1998). Aquatic cyanobacteria are known to grow in disparate environments such as freshwater, stagnant ponds, waste water and coastal water. Fluxes in environmental conditions operative in summer or in autumn may allow the proliferation of cyanobacteria to dominant population among phototrophic microorganisms. This may occurs when four conditions namely, i) an absence of wind, ii) a water temperature between 15 and 30°C, (iii) a pH between 6 and 9 and (iv) a relatively rich nitrogen and phosphorous medium exist (Carmichael *et al*., 1994). Other conditions may also influence this propagation such as water oxygenation, a hollow depth and a low light intensity (Skulbert *et al*., 1984). In Tunisia, water reservoirs are the main source of drinking water. Furthermore, nutrient loading coupled with year-round warm weather favor the growth of cyanobacteria in these reservoirs, several of which can produce cyanotoxins, especially microcystins. In order to evaluate seasonal variations of MCYST concentrations in raw water of the reservoir Hjar in the Cap Bon region (North-East Tunisia); monthly sampling was performed during July to December 2003. Many physicochemical and biological parameters of water and cyanobacterial genera present in this reservoir were identified. The protein phosphatase inhibition assay (PP2A) was employed to determine microcystin concentrations in the raw water. Furthermore, we characterized different MCYST congeners present in the cyanobacterial blooms by high performance liquid chromatography coupled to UV and tandem mass spectrometry.

Methods

Raw water and phytoplankton sampling

During the period of study (July – December 2003), raw water and phytoplankton samples were collected monthly from the same site at the shallow reservoir, in the Cap Bon region of Tunisia (Fig. 1). The volume water of this reservoir is 5.5 Mm^3, with a maximum of depth of 6 Meters. This reservoir is used principally for irrigation of market gardening (lettuce, tomatoes, peppers, etc…).

Phytoplankton samples were harvested from the water surface and fixed with formaldehyde and Lugol at 1 % and 0.2 % (v/v) final concentration, respectively. The cyanobacteria genera were determined according to taxonomic keys based on cell structure and dimension, colony morphology, and mucilage characteristics (Geitler, 1932, Komarek & Anagnostides, 2001). For microcystin analysis, raw water samples were collected between 0 and -0.5 m and stored at 4°C until further processing in the laboratory. Water samples (300 mL) were filtered through glass micro-fiber filters (GF/C, Whatman) to separate toxins dissolved in the water (filtrates) and toxins associated with cyanobacteria cells and/or adsorbed on particles (filters).

Physicochemical water quality analysis

The water temperature and pH were measured directly in the reservoir with portable instruments (WTW model). The dissolved oxygen was measured according to the chemical method of Winkler. The nutrients (nitrate, nitrite, ammonium ion and orthophosphate were measured by methods described in Rodier (1996).

Biological water quality analysis

Chlorophyll-*a* was extracted using methanol and measured with a fluorometer (Turnerdesigns model) according to the method described by Neuveux (1974). The phytoplankton taxa were counted in sedimentation chambers using an inverted microscope (Leitz) according to the method described by Utermöhl (1958).

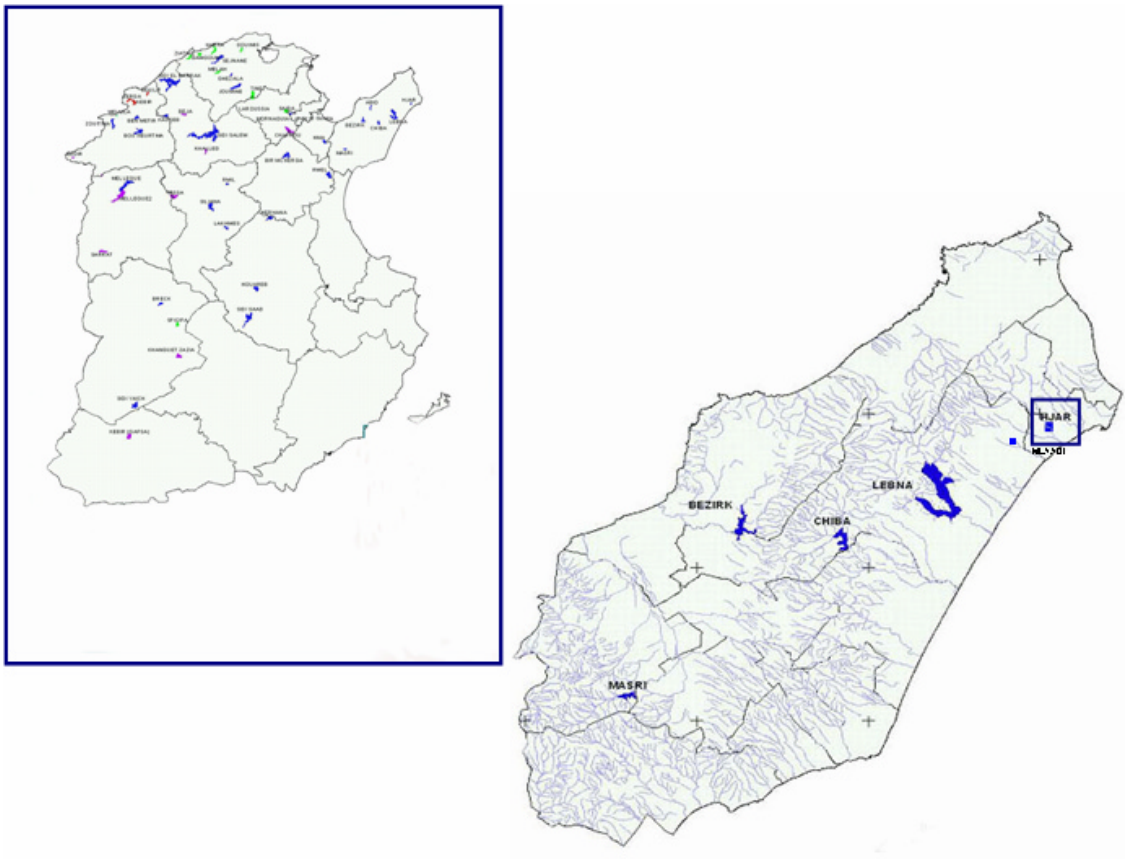

Figure 1. Geographic situation of Hjar reservoir

Extraction and analysis of microcystins by protein phosphatase assays

Filters were extracted (overnight at 4°C) with 10 mL of 80 % (v/v) aqueous methanol. Extracts were filtered through glass micro-fiber filters (GF/C, Whatman) and supernatants were then evaporated to dryness under vacuum. The dry residues were dissolved in 300 µL of methanol. Triplicate aliquots of each sample were then analyzed by the PP2A inhibition assay. However, filtrates were pre-concentrated on a C18 Bakerbond SPE cartridge (Baker, Netherlands) according to the method described by Maatouk *et al*. (2002). The toxin fraction in each eluate after solid phase extraction was then evaporated to dryness under nitrogen and dissolved in 300 µL of methanol. Triplicate aliquots of each sample were then analyzed by the PP2A inhibition assay according to Bouaïcha *et al*. (2002). The total microcystin concentration in the reservoir water was calculated by adding together particulate (intracellular) and dissolved (extracellular) microcystin concentrations.

Identification of microcystin variants by HPLC-PDA and HPLC/MS/MS

An aliquot of the filter extract (November 2003) was analyzed on a HP1090 LC system (Agilent Technologies, France) coupled to a diode array detector. The analytes were chromatographed on a Chrompack C18 (150 x 2 mm i.d., 5 µm) column using water with 0.05 % trifluoroacetic acid (solvent A) and acetonitrile (solvent B) as mobile phase. The gradient program was set: 0 min 80 % A; 10 min 60 % A, 15 min 40 % A, 20 min 20 % A, and 25 min 0 % A; flow-rate 0.5 mL min^{-1}. ESI-MS/MS in positive mode experiments, for confirming the identity of some MCYST-LR variants were performed on a Quatro Premier LC/MS/MS system (Waters, France) and an Alliance 2695 series LC system (Waters, France). The analytes were chromatographed on an ABZ plus C18 (100 x 2.1 mm i.d., 3 µm) column using water (solvent A) and acetonitrile (solvent B) with 0.428 %

hexafluorobutyric acid as mobile phase. The gradient program was set: 0 min 100 % A; 12 min 60 % A, 17 min 30 % A, 27 min 30 % A, 28 min 100 % A, and 45 min 100 % A; flow-rate 0.2 mL min^{-1}. The tandem mass spectrometry was run in multiple reaction monitoring (MRM) mode. The monitored toxin variants and their corresponding m/z were MCYST-YR (1044.62, 602.8), MCYST-LW (1024.29, 1007.5), MCYST-LR (994.63, 976.3), MCYST-LF (985.45, 968.07), and MCYST-RR (518.97, 505.36).

Result

Monthly variation of physico-chemical and biological parameters

Changes in the physico-chemical and biological parameters were investigated for 6 months, from July to December 2003, in the reservoir Hjar. The water temperature, which is an important factor in supporting algal growth, varied from 28.3 °C in the summer and gradually decreased in the autumn to 12.4 °C in the winter. The values of the pH do not show important variations. The reservoir was very well aerated during the survey with average dissolved oxygen levels of 7.04 to 9.79 mg L^{-1}. Total nitrogen concentrations ranged from 3.54 to 20.10 mg L^{-1}. The temporal evolution of the nitrogenous compounds, relative to the addition of nitrate, nitrite and ammonium ions, shows that nitrate representing a proportion varying between 81 and 98 % of the total mineral nitrogen. However, nitrite and ammonium ions present only 1 and 7 % of the total mineral nitrogen, respectively. Total phosphorus concentrations were present in small quantity in the reservoir (0.02 to 0.09 mg L^{-1}) and were largely presented by the ion orthophosphates with 69 % of the total phosphorus. The chlorophyll-a concentrations were very low varying within a range of 0.72 to 4.94 µg L^{-1} and reflected the low density of the phytoplankton in the reservoir. In summer, *Chlorophyceae* and *Diatomophyceae* were predominant groups and from the month of August until November 2003 an increase of cyanobacterial abundance was observed which was composed of *Oscillatoria* spp. and *Pseudoanabaena* sp. The species *Oscillatoria* spp. were present in August and November with a density of 42,000 and 146,000 filaments L^{-1}, respectively and co-occurred with a few filaments of *Pseudoanabaena* sp. However, the species *Pseudoanabaena* sp. was present only in autumn in September, October, and November with a density of 39,000; 238,000; and 42,000 filaments L^{-1}, respectively. In December, the phytoplankton density was very low and cyanobacteria were not observed.

Monthly variation of cyanotoxins type microcystins

The MCYST concentrations in water and algal samples were analyzed by the PP2A inhibition assay. The seasonal variation of the MCYST concentrations in the particulate (intracellular) and in the dissolved (extracellular) samples is shown in figure 2. Measurable MCYST levels were detected in dissolved and particulate fractions in all the examined samples with concentrations ranging from 16.4 to 849.0, and from 1.5 to 6565.8 ng L^{-1}; respectively. High concentration of cell-bound MCYSTs was found in late autumn in November 2003. However, dissolved MCYST concentrations, in all cases except those where cyanobacteria cells are obviously breaking down (July and August) are lesser than the particulate MCYST concentrations with proportions ranging from 4 to 97 % of the total MCYST concentrations. During the six-months monitoring program from July to December 2003, the total MCYST concentrations ranged between 23.4 and 7455.2 ng L^{-1} (Fig. 2). Maximum value was observed in November with concentration in water at 7455.2 ng L^{-1}.

Microcystins identification by HPLC-PDA and HPLC/MS/MS

The HPLC-PDA separation of microcystins present in the water sample in November 2003, where the highest microcystin concentrations are observed, is shown in figure 3A.

The chromatogram of this sample revealed the presence of five peaks having characteristic

spectra of microcystins with a maximum of absorbance at 238 nm.

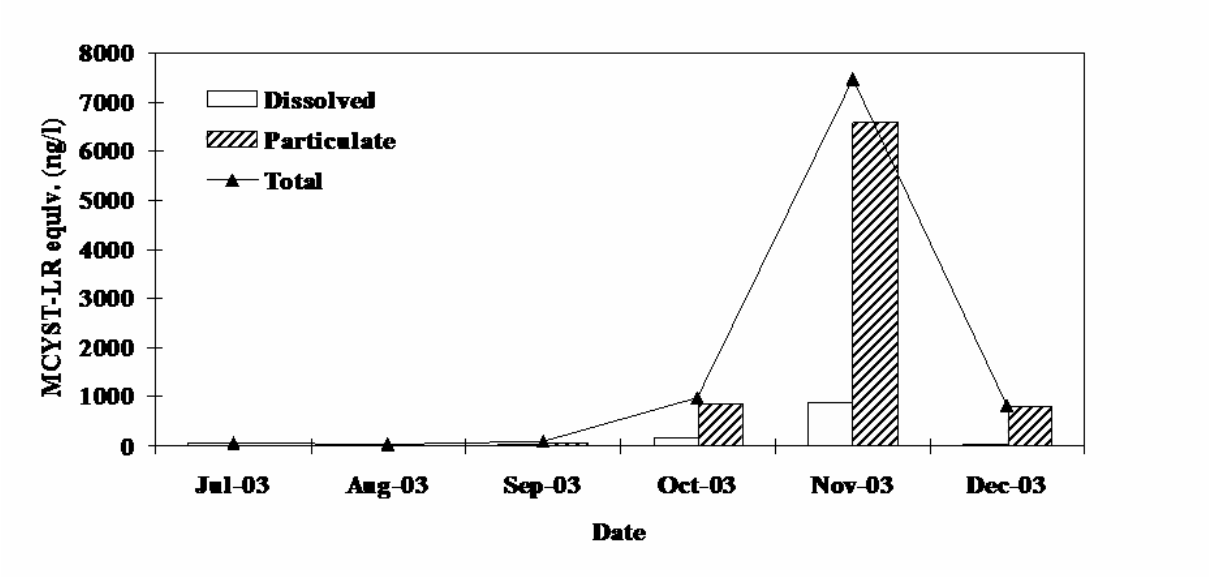

Figure 2. Seasonal variations of dissolved, particulate, and total MCYST-LR equivalents calculated from protein phosphatase inhibition assays in the raw water of the Hjar (Tunisia) reservoir.

HPLC/MS/MS analysis of this sample assigned that peaks 1, 2, and 3 as microcystin-RR (MCYST-RR), microcystin-YR (MCYST-YR), and microcystin-LR (MCYST-LR), respectively by the presence of fragment ions that are known to be indicative of these microcystins (Fig. 4A) as indicated in the standard spectra (Fig. 4B). However, no structural assignments of the peaks 4 and 5 could be made, due to the lack of available standards.

Discussion

Freshwater blooms of toxic cyanobacteria have been reported worldwide (Sivonen & Jones, 1999). In Tunisia, however, there is no data concerning MCYST-producing cyanobacteria. We now report for the first time the presence of MCYSTs from Reservoir Hjar in the Cap Bon region, North-East of Tunisia. The massive development of algae is common in warm countries and in eutrophic reservoirs. Within the North-African basin, In Egypt (Mohamed et al., 2003), in Morocco (Oudra *et al*., 2001), and in Algeria (Nasri *et al*., 2004), microcystins have been detected in reservoirs during the warmest months. However, In Tunisia in the reservoir Hjar the highest concentration of microcystins was observed in late autumn in November 2003. The cyanobacterial community in this reservoir was characterized by the dominance of filamentous species, *Oscillatoria* spp. and *Pseudoanabaena* sp. Nevertheless, in a recent study, Sabour *et al*. (2002), and Nasri *et al*. (2004) showed that in Morocco, and in Algeria, respectively a neighbouring country with close climatic conditions, natural cyanobacterial blooms containing microcystins were dominated by the genus Microcystis. In Egypt, however, isolation and characterization of MCYSTs were reported from *Microcystis aeruginosa* (Abdel-Rahman *et al*., 1993) and *Oscillatoria tenuis* (Brittain *et al*., 2000).

During the period of investigation (July - Decemberr 2003), the total MCYST concentrations ranged between 23.4 and 7455.2 ng L^{-1} MCYST-LR equivalents in the reservoir water (Fig. 2). Thus, the highest microcystin content present in the Tunisian freshwaters was lesser than that reported in Algeria (Nasri *et al*., 2004), and in Morocco (Oudra *et al*., 2001). This could be explained therefore by the

absence of surface scums of cyanobacteria formed during the survey period. The overall density of cyanobacteria was not very high and varied between 0 and 146,000 and 0 and 238,000 filaments L^{-1} for the species *Oscillatoria* spp. and *Pseudoanabaena* sp., respectively.

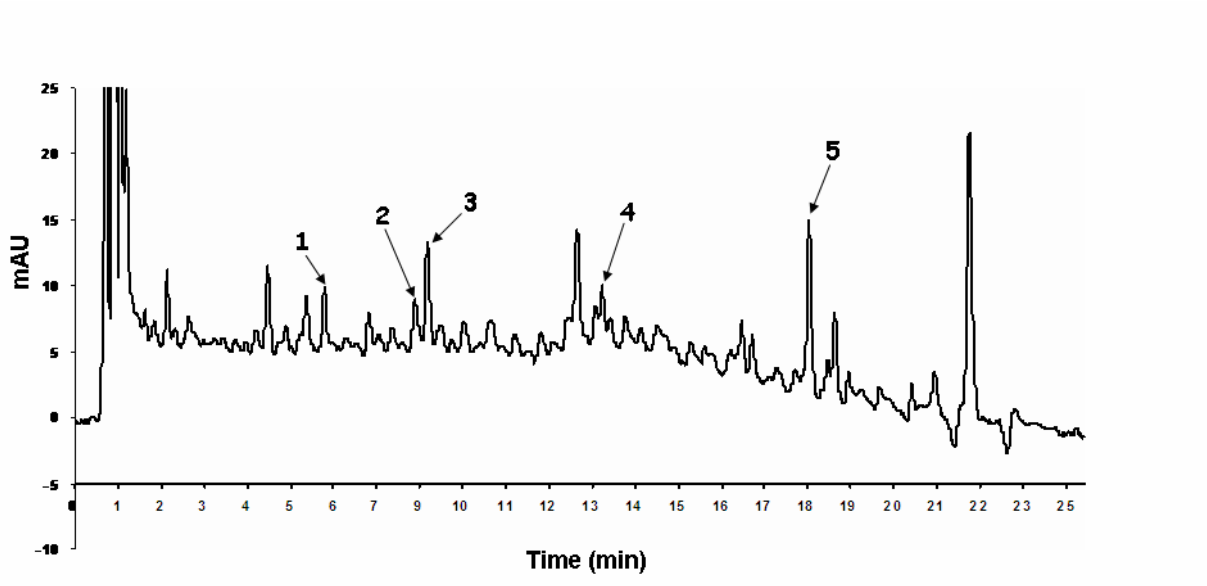

Figure 3A

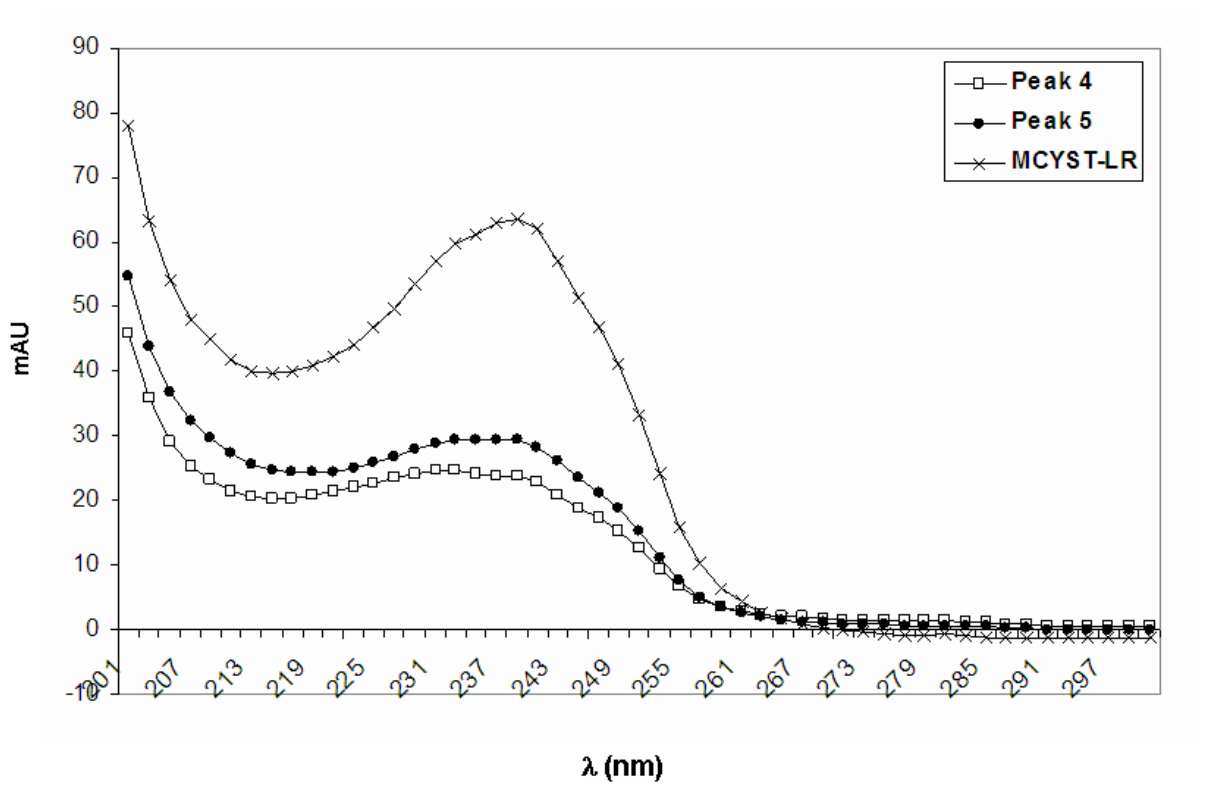

Figure 3B

Figure 3. LC-PDA chromatogram (A) of the crude extract of the cyanobacteria bloom sample (September 2005) harvested from the reservoir Lebna (Tunisia). (B) : UV spectra of the MCYST-LR standard and peaks LB observed in the chromatogram 2.

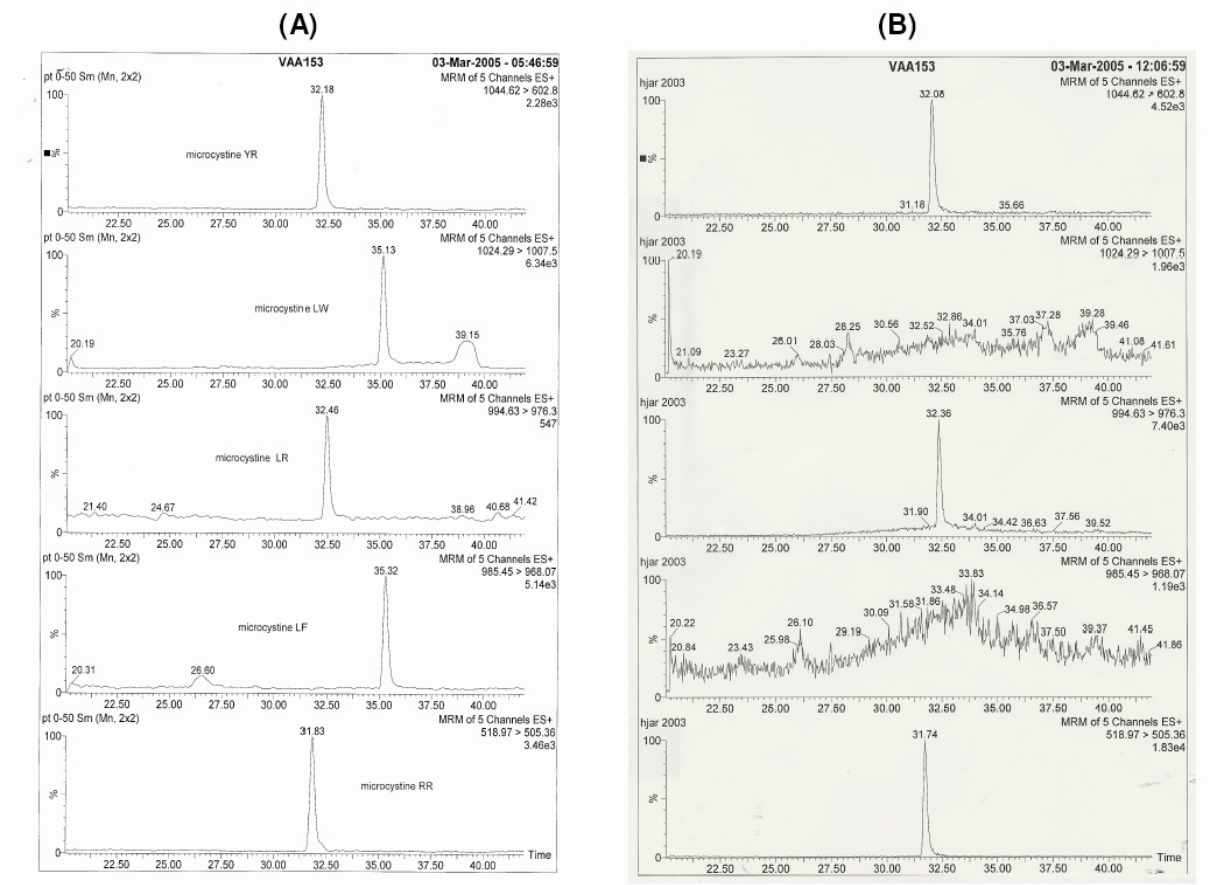

Figure 4. Mass spectrometric spectra of A: microcystin standards (Microcystin-YR, Microcystin-LW, Microcystin-LR, Microcystin-LF, and Microcystin-RR) and B: the crude extract of the cyanobacteria bloom sample (November 2003) harvested from the reservoir Hjar (Tunisia).

It is a fact that the genus *Oscillatoria* do not usually form scums on the water surface since they require lower light intensities for growth, but they may occur in high concentrations deeper in the water column (Lindholm *et al.*, 1989, Lindholm & Meriluoto, 1991).

The crude extract of the cyanobacteria population was analysed by HPLC-PDA on one occasion in November 2003, when protein phosphatase results showed maximal concentrations of cyanotoxins. The chromatogram of this sample showed five peaks having characteristic spectra of microcystins with a maximum of absorbance at 238 nm (Figs. 3A and B). Peaks 1, 2, and 3 (Fig. 4A) were assigned by mass spectrometric detection (HPLC/MS/MS) as MCYST-LR, MCYST-YR, and MCYST-RR variants. However, peaks 4, and 5 although having characteristic spectra of microcystins (Fig. 4B), no structural assignments have been made for there, due to the lack of available standards. Consequently, these unidentified congeners are more hydrophobic than MCYST-LR and peak 5, is the predominant variant (Fig. 3A). The microcystin patterns of the bloom samples observed in Tunisian freshwaters are fairly similar to those reported in Morocco (Oudra *et al.*, 2001), and in Algeria (Nasri *et al.*, 2004) a neighbouring country where MCYST-LR, MCYST-YR, and MCYST-RR are also the most common microcystin. As reported in several studies from Mediterranean countries, MCYST-LR is major toxin in cyanobacterial blooms from Portugal

(Vasconcelos *et al*., 1995, 1996), France (Vezie *et al*., 1997), and Morocco (Oudra *et al*., 2002) and frequently co-occurs with MCYST-YR and -RR in Morocco (Oudra *et al*., 2001). However, in this study and if we considerate that peak 5 (Fig. 4A) is a microcystin-congener, the intensity of peak 2 corresponding to MCYST-LR is not the major variant in cyanobacterial extracts from the reservoir Hjar.

Although the reservoir Hjar is used principally for irrigation of market gardening, the development of cyanobacteria-producing microcystins pose a human health risk. As reported previously, the microcystins present in water destinated for irrigation may not only affect plants growth and development, but may also accumulate in plant tissues (McElhiney *et al*., 2001). It has been shown that lettuce leaves may contain both cyanobacteria and microcystins after irrigation with contaminated water (Codd *et al*., 1999a, 1999b). Although the most usual route of human intoxications is drinking contaminated water, the accumulation of toxins in vegetables may increase the number of intoxications and heighten the long-term effects, including the risk of hepatocancer (Falconer & Humpage, 1996). In addition, cyanobacteria (blue–green algae) are prokaryotic photosynthetic organisms found in a wide range of habitats. These organisms are especially common in freshwater and brackish environments all over the world where, depending on the environmental conditions they can occur in large densities forming blooms (Whitton and Potts, 2000). Nodularin accumulation in edible blue mussels (*Mytilus edulis*) and microcystins in edible catfish (*Ictalurus punctatus*) have been found (Falconer *et al*., 1992; Zimba *et al*., 2001). Therefore, the contact of freshwater cyanobacteria with marine animals is not an uncommon situation and may occur in transitional water ecosystems. In fact, transitional water ecosystems may play an important role on the transfer of cyanobacteria toxins through food chains. Therefore, the presence of these hepatotoxins in Tunisian freshwater led us to suggest that monitoring programs of cyanobacteria and their toxins should be implemented.

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