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Seasonal occurrence and toxicity of *Microcystis* spp. and *Oscillatoria tenuis* in the Lebna Dam, Tunisia

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ABSTRACT

Physicochemical and biological water quality, including the total microcystin concentrations, was investigated for the first time from January to December 2005 in the Lebna Dam, Tunisia. Microcystin levels and characterization of the different microcystin variants present were measured by protein phosphatase (PP2A) inhibition assays and by LC/MS/MS, respectively. Nutrient values were high, with total inorganic nitrogen and phosphorus concentrations ranging from 0.05 to 8.4 mg L^{-1} and from 0.03 to 1.37 mg L^{-1}, respectively. However, the chlorophyll-*a* concentrations were very low with a peak (5.32 μg L^{-1}) on 20 September 2005 at 9 m depth water samples. Microscopic examination of the phytoplankton samples showed the dominance in the autumn of three morphospecies of the genus *Microcystis* and the species *Oscillatoria tenuis*. The total (particulate and dissolved) microcystin concentrations at the surface and at 9 m depth water samples ranged between 0.008 and 1.73, and 0.005 and 5.57 μg microcystin (MC)-LR equivalent L^{-1}, respectively, with a peak on 20 September. The presence of the microcystin synthetase genes (*mcyA*, *-B*, and *-C*) in the lysates of the three morphospecies of the genus *Microcystis* and the species *O. tenuis* indicated that these species were responsible for the microcystin production in this system. The analysis of the field cyanobacterial sample extract containing these species by LC/MS/MS revealed the presence of two microcystin variants: microcystin-LR (MC-LR) and microcystin-YR (MC-YR).

1. Introduction

Toxic blooms of cyanobacteria (blue-green algae) have been observed in different regions of the world (for an overview, see Sivonen and Jones, 1999). The presence of these toxic microorganisms in water bodies used either for drinking or irrigation or for recreational purposes may present serious health risks for the human population. Several species are able to produce potent toxins that cause acute mortality in animals (Carmichael and Falconer, 1993) and illness in humans (Kuiper-Goodman et al., 1999) or, when exposed through hemodialysis, even death (Jochimsen et al., 1998). These health hazards have led the World Health Organization (1998) to establish a provisional guideline value for microcystin (MC)-LR of 1 μg L^{-1} in drinking water. Microcystins are the most widespread of the cyanobacterial toxins. They are hepatotoxic cyclic heptapeptides, with over 70 natural structural variants (Codd et al., 2005), and are potent and specific inhibitors of protein phosphatases (Mackintosh et al., 1990). Microcystins can be produced by *Microcystis* spp., *Anabaena* spp., *Nostoc* spp., *Oscillatoria* spp., and *Planktothrix* spp. (Sivonen and Jones, 1999). Populations of these

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species are known to include both microcystin-producing and -non-producing strains (Vezie et al., 1998; Rohrlack et al., 2001). Several studies reported that freshwater cyanobacteria blooms are typically associated with eutrophic and poorly flushed waters (Paerl, 1988; Carmichael, 1995; Lee et al., 2000; Oliver and Ganf, 2000; Albay et al., 2003). For example, an increase in nutrient loads from either agricultural run-off or sewage treatment plants results in extensive populations of bloom-forming cyanobacteria (George and Bradby, 1993; Humphries and Robinson, 1995). Moreover, Steinberg and Hartmann (1988) and Steinberg and Gruhl (1992) suggest that eutrophication, especially by phosphorus, often leads to significant shifts in phytoplankton species' composition towards bloom-forming cyanobacteria. In Tunisia, 70% of drinking water comes from surface water. Furthermore, nutrient loading from agriculture coupled with year-round warm weather may, therefore, favor the growth of cyanobacteria in these freshwater bodies, several of which can produce cyanotoxins, especially microcystins.

The purpose of this study was to evaluate seasonal occurrence and toxicity of *Microcystis* spp. and *Oscillatoria* sp. in the Lebna Dam in the Cap Bon region (North-East Tunisia) that is used intensively for irrigation and provides drinking water for domestic animals. This dam was sampled twice per month from January to December 2005 to evaluate the fluctuation of microcystin concentrations in the raw water. The protein phosphatase (PP2A) inhibition assay was employed to determine microcystin concentrations in the raw water. PCR technique was used to assess the presence of microcystin synthetase genes (*mcy*A, -*B*, and -*C*) in isolated colonies of the three morphospecies of *Microcystis* and filaments of *Oscillatoria* sp. Furthermore, we characterized different microcystin congeners present in the field sample extract of these species by LC/MS/MS.

2. Materials and methods

2.1. Study site and sampling

The Lebna Dam is located in the Cap Bon region (North-East) of Tunisia with a 650 ha area. During the investigated period, it had a volume of $30.2 \times 10^6\,m^3$, with a maximum of depth of 10 m and a mean depth of 6 m. This dam provides irrigation water for market gardening (lettuce, tomatoes, peppers, etc.) and arboriculture, and drinking water for domestic animals. For phytoplankton identification and pigment, nutrients, and microcystin analysis, water samples were collected twice per month at a single site near the dam outlet from January to December 2005. Water samples were collected using a vertical water sampler (Wildco model) of 2 L capacity from the surface and near the bottom at 9 m depth, and then stored immediately in a portable refrigerator (around 4 °C) and then transported to the laboratory for analysis.

2.2. Phytoplankton analysis

Phytoplankton samples collected in 1000 mL plastic bottles were fixed with formaldehyde and Lugol at 1% and 0.2% (v/v) final concentration, respectively, until analysis. An aliquot of 25 mL was transferred to a sedimentation chamber, and the phytoplankton taxa were then counted using an inverted microscope (Leitz) using the method described by Utermöhl (1958). The cyanobacteria genera were identified using universally accepted taxonomic keys based on cell structure and dimension, and colony morphology, and mucilage characteristics (Geitler, 1932; Komárek and Anagnostidis, 1999, 2005). The other phytoplankton groups were identified according to morphological features described in Bourrelly (1972, 1981, 1985).

2.3. Chemical and physical parameters

Water temperatures and pH were measured *in situ* using a multi-parameter probe (WTW model). The dissolved oxygen was measured using the chemical method of Winkler. Transparency was measured with a Secchi disk. For nutrient analysis, collected raw water samples were shaken vigorously and aliquots were then used to analysis of nitrate (NO_3^--N), nitrite (NO_2^--N), ammonium (NH_4^+-N), orthophosphate (PO_4^{3-}-P), and total phosphorus (TP) using the methods described in Rodier (1996). Phosphorus was measured by the ascorbic acid method. TP was analyzed by the digestion of persulfate method. Concentrations of NH_4^+-N, NO_3^--N, and NO_2^--N were determined using Nessler, sulfosalicylic acid, and Zambelli reaction methods, respectively. The total inorganic nitrogen concentration was then calculated as the sum of nitrate, nitrite, and ammonium nitrogen concentrations. For chlorophyll-*a* determination, the raw water samples were shaken vigorously and an aliquot of 5 mL was filtered through a glass microfiber filter (GF/C, Whatmann), and the filters were then extracted in 5 mL of methanol. Chlorophyll-*a* was then detected fluorometrically using a Turner Designs model fluorometer with a 630 nm excitation filter and 660 nm emission filter according to the method described by Neuveux (1974).

2.4. Sample preparation and microcystin analysis by the PP2A inhibition assay

For analysis of microcystins, an aliquot of 300 mL of water samples, collected in glass bottles at the surface and at 9 m depth, was filtered through a glass microfiber filter (GF/C, Whatmann) to separate out the toxins dissolved in water (dissolved toxins) and those associated with cyanobacterial cells and/or adsorbed on particles (particulate toxins). The filters were extracted with 10 mL of 80% (v/v) methanol/water, and the filtrates were then pre-concentrated over a Baker-bond SPE cartridge (Baker, Netherlands) as described by Maatouk et al. (2002). The toxin fraction in each eluate after solid-phase extraction, and in each methanol filter extract were then evaporated to dryness under nitrogen, and dissolved in 300 μL of 50% (v/v) aqueous methanol. Aliquots of each sample were then analyzed using the PP2A inhibition assay according to Bouaïcha et al. (2001) with minor modifications. Briefly, PP2A activity was determined by measuring coloration associated with the formation at 37 °C of *p*-nitrophenol (*p*-NP) from the substrate *p*-nitrophenyl phosphate (*p*-NPP) using a microtiter plate reader (Bio-Tek Instruments, ELX800G). *p*-NPP (80 mM) was dissolved in a buffer containing 40 mM Tris–HCl, 34 mM $MgCl_2$, 4 mM EDTA,