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Toxicity assessment and detection of cyanobacterial toxins (Microcystins) in a Mediterranean natural lake (Dayete Aoua, Morocco)

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Abstract

Dayet-Aaoua lake (DA) is a freshwater aquatic ecosystem of ecological interest, located at a middle Atlas mountains under humid hydrographic basin (Ifran, Morocco) (33° 39′ 10″ N, 5° 02′ 30″ W). This work deals on the toxicity assessment of *Microcystis aeruginosa strain* isolated from DA lake, using both *Artemia* bioassay and the high-performance liquid chromatography (HPLC) equipment provided with a photodiode array detector (PDA. HPLC-PDA identification and quantification of hepatotoxins cyanotoxins (Microcystins). The toxicity of the cultural *Microcystis* biomass evaluated by *Artemia* bioassay revealed a positive letal concentions (LC) (24-h LC_{50} = 9.01 mg mL^{-1} and 40-h LC_{50} = 6.87 mg mL^{-1}). Microcystins (MC) variants selected by HPLC-PDA showed a high MC content concentration (185.56 µg g^{-1} Dry weight) which confirmed the toxicity assessed. Four MC variants were clearly identified in the DA biomass (MC-WR, MC-RR, DM-WR, MC- YR). For the first time in this natural lake, the obtained results confirms the presence of producing cyanotoxins *Microcystis* strains, detection of various microcystin congeners and quantification of high amount of MCs. In such ecological ecosystem, the presence of toxic *Microcystis*, especially in favorable periods of cyanobacteria bloom proliferations, may be regarded as an environmental threat and health hazards. Therefore, an establishment of cyanotoxins monitoring program in this natural lake is highly recommended.

1. Introduction

Natural lakes are widely distributed in Mediterranean biogeographic areas. These aquatic environments, considered as special wetlands of ecological interest, constitute rich and particular biotopes for animal and plant biodiversity [1]. However, the biodiversity of these ecosystems suffers pressures due to over-human exploitation and the proliferation of invasive organisms [2].

In these aquatic environments, cyanobacteria, among others autotrophic microorganisms play the principal role as primary producers. They live in abundance with relatively high temperature and micronutrients richness [3]. While, these microorganisms are known by producing a wide range of toxic compounds, named ”cyanotoxins”; including hepatotoxins, neurotoxins and dermatotoxins [4, 5] which are harmful to livestock, aquatic animals, birds and humans [6]. Cyanobacterial toxins can be classified according to their chemical structures as cyclic peptides (microcystin and nodularin), alkaloids (anatoxin-a, anatoxin-a(s), saxitoxin, cylindrospermopsin, aplysiatoxins, lyngbyatoxin-a) and lipopolysaccharides [7]. They are potent liver toxins, general tumor promoters, inhibitors of protein phosphatases and inhibitors of protein synthesis [8, 9].

The hepatotoxic microcystins are common cyanotoxins [4]. MCs are produced by various cyanobacterial genera, including *Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis* [10]. More than 100 structural microcytsin congeners have been identified to date [11].

In Morocco, as a Mediterranean country, most of natural lakes are widely spread in the Middle Atlas Mountains, especially under wet climate and forest areas [12]. According to cyanobacteria monitoring studies, most of investigations were carried out in reservoirs used for drinking water supplies where *Microcystis* cyanobacteria blooms have appeared regularly in summer and autumn [13, 14]. Cyanobacteria prospection in divers natural lakes ecosystem of ecological interest are very limited and the occurrence of toxic cyanobacteria strains producing cyanotoxins in such aquatic environments (e.g. natural lakes) has not been seriously taking into consideration and the potent sanitary health risks has not profoundly assessed thus far.

This present work focused on the occurrence of *Microcystis aeruginosa* cyanobacteria strains producing some hepatotoxins Microcystins congerners in DA lake and the toxicity assessment of its cells extract using both the *Artemia* bioassay and the detection of Microcystins by HPLC-PDA analysis.

2. Materials and methods

2.1. Study area and General Characteristics

DA lake is a freshwater aquatic ecosystem of ecological interest, located at a middle Atlas Mountains (33° 39′ 10″ N, 5° 02′ 30″ W), at 15km from of Ifrane city. It is a permanent surface freshwater natural lake, with an important area of 140 Ha. The lake is surrounded by mixed forests of oaks and cedars, dominated by *Quercus rotundifolia* and *Cedrus Atlantica*. The submerged and emergent aquatic plants are especially dominated by "*Myriophyllum spicatum, Juncus bufonius, Polygonum amphibium, Ranunculus millifoliatus, Scirpus lacustris"*. This vegetation characterizes the sub and humid bio-climate. The lake is also known by a wide variety of wildlife: fishes (*Micropterus salmoides, Percafluviatilis, Tincatinca..), birds (Coots hooded, Anasclypeata, Anasplatyr hynchos, Anasstrepera* ..), and also a special macro fauna (over 20 species of planktonic crustaceans). It is classified as an aquatic systems of high biological and ecological value protected by the RAMSAR convention.

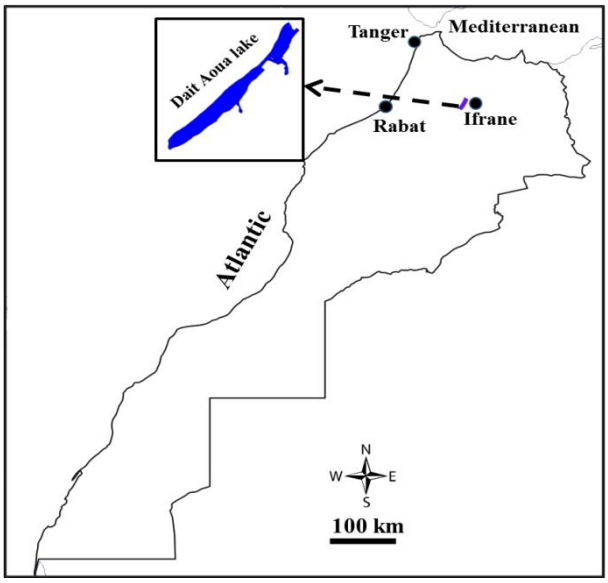

Figure 1: Geographic localization of DA Lake

2.2. *Sampling and analysis*

Biomass from planktonic cyanobacteria was collected with a 27 µm mesh phytoplankton net at the natural lake (DA). Z8 medium [15] was used for the isolation and culture of the *Microcystis* cyanobacteria. Cultures were maintained in laboratory at 25 °C ± 2°C, at a light intensity of 82 $\mu E.m^{-2}.S^{-1}$ fluorescent continuous light and with a light / dark cycle of 16h / 8h. The cyanobacterial biomass produced was harvested at the end of the exponential growth phase after flotation or by centrifugation. The concentrated biomass was directly used or freeze-dried and stored at – 20 °C.

2.3.*Artemia salina bioassay*

The brine shrimp *Artemia salina* is used extensively for toxicity bioassay, since MCs have been shown to be highly toxic to this organism [16]. The dried brine shrimp eggs were purchased from special stores. The larvae (18-20 in each well of a 96-well microtiter plate) were used in tests 24h after hatching. Lyophilized biomass cells were sonicated and prepared for 5 final concentrations (10, 5, 2.5, 1.25, 0.625 mg mL^{-1}). These toxic fractions were pre-purified from 75% methanol extracts by using an activated Octadecyl Silica - ODS gel cartridge. The obtained extract was evaporated and resuspended in distillated seawater. The method followed was essentially that described by [17]. The toxicity of cyanobacterial fractions to *A. salina* larvae was tested in natural seawater, in loosely covered micro-plates at 25°C. The test results were expressed as percentage of dead individuals [18], after 24 and 40-h of exposure to biomass extracts. LC_{50} (µg D.W of cultural biomass mL^{-1} to attain 50% lethality) was estimated by EPA Probit Analysis Program (Version 1.5).

2.4. *Detection and quantification of microcystins by HPLC-PDA*

2.4.1. Microcystin extraction

Lyophilized biomass was extracted with 70% aqueous methanol (2mg DW mL $^{-1}$) and dried by rotary evaporation at 45ºC. Two extract types were obtained as follows: concentrated extract, by extracting twice the biomass with 150 µL of 70% methanol; and dilute extract, by extracting with 500 µL of 70% methanol the residue of the biomass extracted with the said 300 µL. This procedure ensures total recovery of microcystins. All extracts were filtered through a GF/C glass filter before being subjected to HPLC.

Standard M-YR, -RR, DM-WR were purchased from Calbiochem (Germany). MC-WR was purified in the laboratory of the Autonomous University of Madrid. Other MCs different from the said ones were quantified using MC-LR as a standard. All chemicals were of chromatographic grade (ScharlauChemie Barcelona, Spain).

2.4.2. HPLC-PDA analysis

Chromatographic analysis of MCs was performed in a HPLC equipment (Waters, model 2695) provided with a photodiode array detector (model 996). The column used was Chromolith C18 (250 mm x 4.6 mm, 5mm.). The mobile phase was a discontinuous gradient of water and acetonitrile, both with 0.05% trifluoroacetic acid. The volume injected was 100 or 50 µL, as advisable, and the mobile phase run at 1mL min^{-1}. MCs were identified on the basis of their UV-spectra and retention time.

3. Results

3.1.*Toxicity assessment*

Toxicity was assessed by *Artemia* and specially two different biomass concentrations were used for the assessment, 10 and 5 mg mL^{-1}. As it appears in Table 1, the mortality rate after 24 h corresponding to the concentration of 10 and 5 mg mL^{-1} were 56.40% and 33.57 %, respectively. Figure **2** shows the effect of fractionated extracts of the cultural *Microcystis* biomass on *A. salina* as well as the mortality calculated at 24 and 40 h. The values of 24 and 40h LC_{50} obtained for the cultured biomass were 9.01 and 6.87 mg mL^{-1}, respectively.

Table 1: Toxicity of cultured biomass of cyanobacteria isolated from DA lake, evaluated by *A. salina* bioassay. Numbers represent percentage of mortality

		percentage of Mortality					
		Extract biomass concentration (mg mL^{-1})					
Extract	Exposure time	10	5	2.5	1.25	0.625	Toxicity
DA cultured biomass	24 h	56.40 **(> 50)**	33.57**(> 20)**	25.35	19.57	9.95	**Toxic**
	40 h	62.41	38.01	28.73	24.88	14.34	

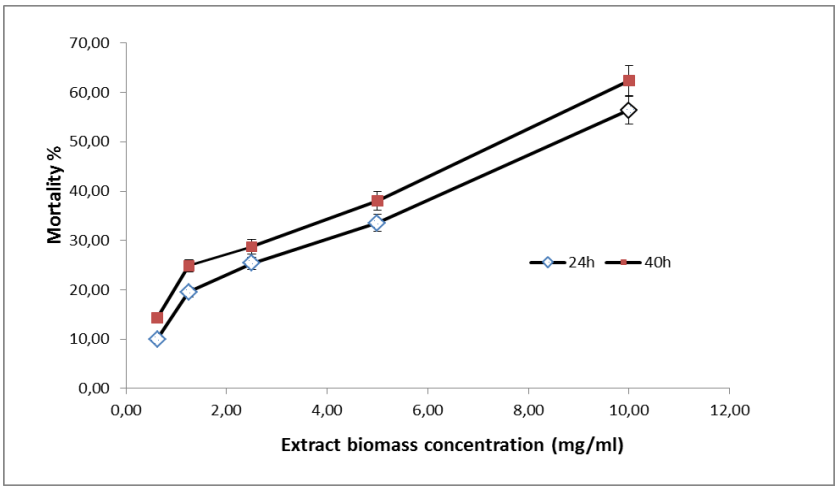

Figure 2: Effect of MC-containing extracts from culture *Microcystis* biomass on *A. salina* after 24h (a) and 40h (b) of exposure

3.2. *Toxicity assessmentIdentification and quantification of Microcystins*

HPLC analysis revealed a clear MC content (as Microcystin- LR (MC-LR) equivalents) in the cultural *Microcystis* biomass (185.56 µg.g^{-1} DW). The chromatogram exhibited 4 MC variants (Fig. 3), that contributed in different degree: WR (56.94), RR (21.1), DM-WR (15.03), YR (6.93).

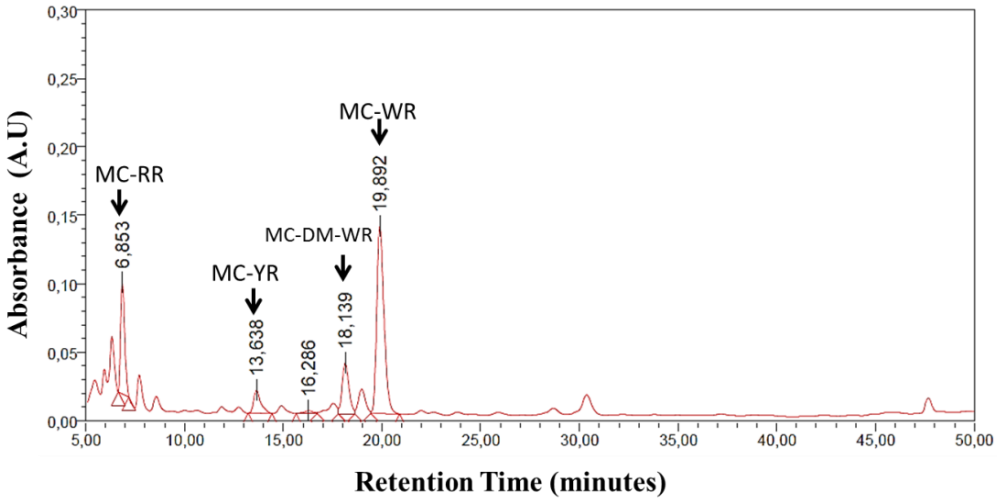

Figure 3: HPLC Chromatograms of *Microcystis* biomass of the DA lake. HPLC conditions are described in Material and Methods.

4. Discussion

According to the biological value of the studied site, the results dealing with the monitoring of potent toxic cyanobacteria strain and assessment of biomass toxicity are very useful. For the first time the assessment of toxicity of the *Microcystis* strain isolated for the DA natural lake, both by *Artemia* bioassays and HPLC-PDA technique, were carried out.

The determination of toxicity given by *Artemia* bioassay shows the values of the mortality rate after 24 h corresponding to the concentration of 10 and 5 mg mL^{-1} were 56.40% and 33.57 %, respectively. These values allows to classify the biomass as toxic, according to [19], who suggested to take as toxic cyanobacteria biomass; samples for which the concentration of 10 mg mL^{-1} causes after 24 h of exposure a mortality of more than 50% of the shellfish larvae, and the exposure to 5 mg mL^{-1} results in the death of more than 20% of the individuals.

The values of 24 h LC_{50} obtained for the cultural biomass was 9.01 mg mL^{-1}. This toxicity could be regarded as lower when compared with those previously reported in the *Microcystis* blooms from Mansour Eddahbi and Almassira reservoirs with the 24h LC_{50} 1.71 mg mL^{-1} and 4.34 mg mL^{-1}, respectively [17]. But slightly bigger than those previously observed in OuedMellah reservoir (6-46 mg mL^{-1}; [20]). Taking into account the toxicity assessed by *Artemia* biotest, it seems that cyanobacteria toxicity assessed from our *Microcystis* culture biomass was lawyer. This can be explain by the changes of the laboratory conditions.

There is a direct relationship between toxicity and MC content in the analysed biomass. The MC concentration in *Microcystis* biomass (185.56 µg.g^{-1}) is remarkably higher than those reported for *Microcystis* strains isolated from the Moroccan reservoir Oued Mellah (0.79-10.48 µg.g^{-1}) [21, 22]. Similar results were observed in other Moroccan ones: Imfout (744 µg.g^{-1}) [23], Takerkoust (130- 650 µg.g^{-1}) [22], Almassira (190- 335 µg.g^{-1}). However, it remains relatively low compared to other works in Mediterranean region: 2100-11300 µg.g^{-1} in Portugal [24], 90-5060 µg.g^{-1} in France [25].

The *Microcystis* biomass was qualitatively diversified with respect to the type of MCs. 4 variants were identified. All of them are mainly reported in Moroccan reservoirs [14]. MC- RR, MC- WR are generally the two predominant Moroccan variants [17]. This dominance seems to be a characteristic of the Moroccan strains grown under normal conditions. The predominance of MC- WR and MC- RR among other MCs variations in this strain confirms although that many cyanobacterial strains can produce several MCs variants, which one or two may be dominant in the same strain [10]. In other countries, the *Microcystis* isolated strains are often dominated by MC- LR: Portugal [24], France [25] and Greece [26].

Conclusions

The obtained results confirm the toxic potential of the *Microcystis* strain isolated from this Moroccan natural lake, located under humid climatic conditions. The biotoxicity assessment, confirmed by *Artemia* biotest shows a positive toxicity. Also, original data, related to MC identification (4 MCs variants) and quantification (185.56 µg g^{-1}) from *Microcystis* biomass were presented. The presence of toxic *Microcystis* in these types of aquatic environments constitutes a real potential factor for the proliferation of *Microcystis* toxic blooms. Therefore the cyanotoxins water contamination.

In this ecosystem known by an ecological interest, the presence of toxic *Microcystis*, especially in favorable periods of cyanobacteria bloom proliferations, may be regarded as an environmental threat and health hazards therefore, an establishment of cyanotoxins monitoring program in this natural lake is highly recommended.

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