First results on *Microcystis ichthyoblabe* Kütz. toxic bloom in the hypertrophic Oued Mellah reservoir (Morocco)

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The Oued Mellah reservoir (33°30' N-07°20' W) is a shallow brackish lake located 25 km North of Casablanca. A study of the phytoplankton community of this lake shows that it is affected by potentially toxic blooms of Cyanobacteria and Haptophyceae. *Microcystis ichthyoblabe* (first time recorded from Morocco), *Anabaena aphanizomenoides*, *Oscillatoria chlorina* (Cyanobacteria) and *Prymnesium parvum* (Haptophyceae) constitute the dominant phytoplankton taxa. *M. ichthyoblabe* starts its growth in late spring. In 1999, it formed a spectacular bloom during May-June, invading the lake with a maximal biomass of 298 mg_{FW}.L⁻¹. This proliferation coincided with high temperatures, strong luminous intensity, alkaline pH and low to undetectable contents of nitrates and orthophosphates. The toxicity of the *M. ichthyoblabe* bloom material was first determined by mouse bioassay. Bloom material collected during the maximal development phase has an LD₅₀ about 502 mg_{DW}.Kg⁻¹. Hepatotoxicity was confirmed by histopathological study. The total contents of microcystins determined by ELISA technique both for bloom material and the isolated strain are about 0.79 and 5.4 µg.g_{DW}⁻¹ respectively. Analysis by HPLC-PDA allows the detection of 11 variants of microcystins. The use of the microcystin-LR authentic sample showed the absence of this microcystin variant in the samples analysed.

Premières données sur un bloom toxique à *Microcystis ichthyoblabe* Kütz. dans le lac-réservoir hypereutrophe Oued Mellah (Maroc)

Mots-clés : blooms, *Microcystis ichthyoblabe*, microcystines, HPLC-PDA, ELISA, Maroc.

Le lac de barrage Oued Mellah (33°30'N-07°20'W) est un réservoir saumâtre peu profond situé à 25 Km au nord de Casablanca. L'étude du peuplement phytoplanctonique montre qu'il est le siège de développement de blooms variés de Cyanobactéries et d'Haptophycées potentiellement toxiques. *Microcystis ichthyoblabe*, signalée pour la première fois au Maroc, *Anabaena aphanizomenoides*, *Oscillatoria chlorina* et *Prymnesium parvum* sont les espèces dominantes. Selon les années, *M.*

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ichthyoblabe prolifère dès le printemps. En mai-juin 1999, cette espèce a formé un bloom spectaculaire envahissant le lac avec une biomasse maximale de 298 mg/PF.L⁻¹. Cette prolifération a coïncidé avec des températures relativement élevées, une forte intensité lumineuse, des pH alcalins et des teneurs faibles voir nulles en nitrates et orthophosphates. La toxicité de *M. ichthyoblabe* a été évaluée par le biotest souris. La DL₅₀ calculée pour un échantillon du bloom en phase de développement maximal est de 502 mg.Kg⁻¹. L'hépatotoxicité a été confirmée par une étude histopathologique. Les teneurs en microcystines totales, déterminées dans le bloom et la souche isolée de *M. ichthyoblabe* par la technique ELISA, sont faibles et respectivement de l'ordre de 0,79 et 5,4 µg.g⁻¹. L'analyse par chromatographie liquide à haute performance avec détecteur à barrette de diodes (HPLC-PDA) montre la présence de 11 variantes de microcystines avec absence de la microcystine-LR en comparaison avec le chromatogramme du standard.

1. Introduction

Blue-green algal blooms are a diagnostic of accelerating eutrophication and are becoming an increasing problem in fresh, brackish and marine waters (Paerl 1996, Falconer 1998). These phenomena have been reported in many countries throughout the world (Skulberg et al. 1984, Carmichael & Falconer 1993) and have been known to cause death in livestock and wildlife besides cases of human illness and mortality (e.g. Jochimsen et al. 1998, Kuiper-Goodman et al. 1999). Cyanobacterial toxins include hepatotoxic peptides, cytotoxic alkaloid, neurotoxic and lipopolysaccharides (Falconer 1999). *Microcystis* is the most cosmopolitan of the cyanobacterial genera (Carmichael 1996) and its blooms and scums are often extensively persistent and, in certain latitudes, are dominant thorough the year (Zohary & Robarts 1990). This genus includes a wide range of toxic species such as *M. aeruginosa* Kütz., *M. wesenbergii* Kom., *M. flos aquae* Elenkin, *M. viridis* Lemm. and *M. ichthyoblabe* Kütz. (Carmichael & Falconer 1993, Watanabe 1996, Falconer 1998). The later species sometimes forms heavy water blooms in mesotrophic and eutrophic waters, but not in polluted water bodies. It commonly occurs in the northern areas of the temperate zone (Komarek 1991). In Morocco, *M. ichthyoblabe* blooms was never recorded before (Loudiki et al., submitted). This paper presents the first results on the dynamics of phytoplankton in the Oued Mellah lake-reservoir, particularly of *M. ichthyoblabe*, the cyanobacterium responsible for spectacular blooms. In addition, data on its toxicity using mouse bioassay, detection and quantification of microcystins by High Performance Liquid Chromatography with Photodiode Array Detection (HPLC-PDA) and Enzyme-Linked ImmunoSorbent Assay (ELISA) techniques are presented and discussed.

2. Material and methods

2.1. Study area and water sampling

Geographic situation and general characteristics of Oued Mellah lake are presented in the figure 1 and table 1. The sampling was carried out at monthly intervals in 1999 and weekly during the bloom periods. Water samples were collected in the station at the deepest part (7-9m) of the reservoir at 0, 1, 2, 4m of depth and near the bottom with a Van Dorn water

Fig. 1. Geographic situation of Oued Mellah reservoir.

Fig. 1. Situation géographique du lac-réservoir Oued Mellah.

Table 1. General characteristics of Oued Mellah reservoir.

Tableau 1. Caractéristiques générales du lac-réservoir Oued Mellah.

Oued Mellah reservoir	
Date of construction	1928-1931
Geographical situation	33°30'N - 07°20'W
River	Oued Mellah
Depth max	9 m
Depth average	4 m
Normal elevation	101,75 NGM
Total capacity	18 Mm ³
Useful capacity	8,8 Mm ³
Surface	250 ha
Catchment surface	1800 km ²
Catchment geology	Primary schist surmounted by greso-maghrebian tile with triassic saliferous clays and altered basalts
Bioclimat	Semi-arid thermomediterranean with hot winter
Use	Domestic water production, irrigation, pisciculture, electricity, sport fishing and recreation
Ecological interest	National Biologic and Ecological Interest Site (B.E.I.S. n° H8)

sampler. Temperature, pH (pH-meter WTW 90), Transparency (Secchi disk), conductivity (WTW LF-90) and incident light (lux-meter LX-101) were measured *in situ*. The other physical and chemical parameters were determined within 24 hours at the laboratory according to the standard methods (Rodier 1984, AFNOR 1994).

2.2. Phytoplankton analysis

For qualitative studies, phytoplankton samples were collected with 40 µm mesh size net and observed with a light microscope for identification. Quantitative samples of 0.5 litre of water were immediately fixed in the field with a neutralised formaldehyde solution. Phytoplankton was counted using sedimentation chambers and an inverted microscope using Utermöhl's technique (1958). The *Microcystis* cells were counted with haematocytometer (Mallassez) after disintegration of colonies by ultrasonication. The

biomass was estimated in fresh weight (mg_{FW}.L⁻¹) from the biovolume of the taxa ; these were calculated by considering the cells as solid geometric shapes (Wetzel & Likens 1991) and assuming that the organisms density was 1g.cm⁻³.

2.3. Toxicity confirmation and microcystins analysis

Natural blooms of *M. ichthyoblabe* was collected by concentrating cyanobacteria during maximal growth of bloom (30/05/1999) with a 40 µm mesh size plankton net. *M. ichthyoblabe* strains were isolated and cultivated in Z8 medium (Hughes et al. 1958, modified by Kotai 1972). Concentrated algal samples of both bloom and strains were lyophilised and stored at -40°C.

The toxicity was measured by intraperitoneal (i.p.) mouse bioassay using 20-30 g male Swiss mice. Sus-

pensions of lyophilised cells in 0.9 % NaCl solution were injected in pairs of mice per dose level. Symptoms were registered and dead animals were observed internally for signs of hepatotoxicity. The toxicity was quantified as LD₅₀ (Lethal Dose for 50 % mortality of tested animals). For confirmation of poisoning signs, liver histopathological examination was performed.

2.4. Immunological detection of microcystins

For toxins quantification, an enzyme-linked immunosorbent assay (ELISA) was performed and polyclonal antibodies microplate Enviro Gard® Microcystins Kit were used.

2.5. High Performance Liquid Chromatography - Photodiode Array Detection (HPLC-PDA)

For the toxins analysis for both *Microcystis* natural bloom and isolated strain, an extraction was required prior to HPLC separation. Toxins were extracted three times with 100 % methanol (Lawton et al. 1994) from lyophilised cells after sonication using an ultrasonic desintegrator (Vibra cell™, Sonics & materials inc.). Extracts were centrifuged (10 min, 4°C, 5000xg) and supernatants were diluted with ultrapure water (Milli-Qplus) down to 20 % methanol. The extracts were loaded in a Bond Elut C18 cartridge (Sep-Pak® Vac C18 6cc, Waters Corporation, Milford USA) previously activated with methanol and water. After washing the column with 20 % methanol and water, the toxic fractions were eluted with 100 % methanol. The fractions were evaporated completely at 40°C (with an evaporator MEYER N-EVAP, USA). Prior to HPLC analysis, the residues were resolved in 50 % methanol and filtered through a 0.2 µm filter. The detection of microcystins was performed with an HPLC system composed of a pump L-7100, a L-7200 autosampler and a L-7450 photodiode array detector (LaChrom Merck HITACHI). Extracts were separated on LiChrospher R 100, 5µm ODS, (LiChroCART® 250-4 cartridge system, Merck, KGaA, Germany) using a gradient of aqueous acetonitrile (with 0.05 % TFA) according to the method of Lawton et al. (1994) with a flow of 1 ml.min⁻¹. UV spectra were obtained from 220 to 400 nm.

3. Results and discussion

3.1. General characteristics of the phytoplankton

The qualitative analysis of the phytoplankton community shows that 69 taxa were inventoried. The spe-

cies *Prymnesium parvum* N. Carter (Sabour et al. 2000) and *Microcystis ichthyoblabe* Kütz. 1843 were inventoried for the first time in Moroccan water bodies. The occurrence of these species does not imply their new apparition in Morocco but rather the lack of previous phytoplankton studies in Oued Mellah lake. Chlorophyceae represented 37 % of the total specific relative abundance, followed by Cyanobacteria (26 %) (Table 2) and Bacillariophyceae (19 %). In spite of the numerous inventoried taxa, the phytoplanktonic seasonality showed that only some species (*M. ichthyoblabe*, *Anabaena aphanizomenoides* f. *forti* and *P. parvum*) are quantitatively important.

The chlorophyll a variation shows that the maximal values 222 and 322 µg.L⁻¹ were respectively recorded in May and September 1999 (Fig. 2a). These values coincided with a maximal development of two algal blooms constituted by *Microcystis* (May-June) and *Anabaena* associated with *Prymnesium* (August-September). The annual average of chlorophyll a is also elevated (75.63 µg.L⁻¹) which allows to classify the Oued Mellah lake in the hypertrophic category.

The phytoplanktonic biomass, expressed in fresh weight, showed a great temporal variation (Fig. 2b). This biomass was very weak in winter (2.9 mg_{FW}.L⁻¹) with dominance of Chlorophyceae (72 %) and Chrysophyceae (22 %) (Fig. 3). The spring was marked by the predominance of Cyanobacteria (97 %), represented essentially by *M. ichthyoblabe*, forming a spectacular bloom during the May-June period. Phytoplanktonic biomass of 300 mg_{FW}.L⁻¹, mainly bound to the development of *M. ichthyoblabe* bloom, constituted the maximal value. In summer, the Cyanobacteria represented again 76.9 % of the total biomass, with a second bloom of *A. aphanizomenoides*. This season was also marked by the beginning development of Chrysophyceae (17 %) represented only by *P. parvum*. In autumn, the weak recorded biomass (7 mg_{FW}.L⁻¹) was constituted essentially by Chrysophyceae (73 %) and Cyanobacteria (21 %).

3.2. Kinetic growth of *Microcystis ichthyoblabe* bloom

Isolated strains and bloom-forming *Microcystis* were identified as *M. ichthyoblabe* (Starmach 1966, Komarek & Anagnostidis 1986, Watanabe 1996, Huber-Pestalozzi 1938). This species is characterized by groups of gathered small cells (average 3 µm in diameter) disposed in fine, colourless and diffuent mucilage. The distribution of cells in colonies is irregular, groups of cells are sometimes localised in a mass of scarcely distributed cells (Komarek 1991).

Table 2. Inventory of planktonic cyanobacteria in Oued Mellah reservoir.

Tableau 2. Cyanobactéries planctoniques inventoriées dans le lac-réservoir Oued Mellah.

Bloom forming-species	
<i>Microcystis ichthyoblabe</i> Kützing	
<i>Anabaena aphanizomenoides</i> Forti	
Other inventoried cyanobacteria	
<i>Microcystis pulvereae</i> f. <i>delicatissima</i> Elenkin	<i>Oscillatoria gracilis</i> Böcher
<i>Synechocystis salina</i> Wislouch	<i>Oscillatoria lauterbornii</i> Schmidle
<i>Synechococcus elongatus</i> Nägeli	<i>Oscillatoria limnetica</i> Lemmermann
<i>Merismopedia</i> sp.	<i>Planktothrix agardhii</i> (Gom.) Anagnostidis &
<i>Phormidium ambiguum</i> Gomont	Komarek [basionym : <i>Oscillatoria agardhii</i>
<i>Phormidium tréleasi</i> Gomont	Gom.]
<i>Lyngbya aestuarii</i> Liebmann	<i>Spirulina major</i> Kützing
<i>Oscillatoria brevis</i> Gomont	<i>Anabaena spiroides</i> Klebahn
<i>Oscillatoria chlorina</i> Kützing	

Conversely to *M. aeruginosa* Kütz. which is the most common species of the genus, data on the geographic distribution, ecology and toxicity of *M. ichthyoblabe* are scarcely reported. For Moroccan waters, we have inventoried this species for the first time in June 1997 in the Oued Mellah lake. It was noted in Europe by Huber-Pestalozzi (1938) in the Baltic sea, in Greece by Gkelis et al. (2000), in Czechoslovakia by Kopp & Hetesa (2000) and quoted by Tilden (1909) in some American lakes. In Japanese lakes, Komarek (1991) reported 26 citations of this species.

The weekly follow-up of the growth (Fig. 4) shows that *M. ichthyoblabe* started its development since the beginning of May. This species presented an exponential growth phase that progresses until the end of May, where the high biomass ($298 \text{ mg}_{\text{FW}} \cdot \text{L}^{-1}$) constituted the first annual peak and represented 99 % of the total phytoplankton biomass. During this phase (05 - 30 May), the net maximal growth rate (μ_{max}) was 0.23 day^{-1} . This phase is immediately followed by a fast declining phase (30 May-18 July). Beyond this decline, *M. ichthyoblabe* persists with a low biomass varying between 5.6 and $20.7 \text{ mg}_{\text{FW}} \cdot \text{L}^{-1}$, and disappears from

the water column at the end of August. The profiles of vertical distribution (Fig. 5) show that *M. ichthyoblabe* especially colonizes surface water layers with very high densities. This distribution is the main cause of the extended blooms (see photo1) that can cover about 50 to 75 % of the total surface of the lake.

The follow-up of environmental parameters (Table 3) during the *M. ichthyoblabe* bloom show that this period was characterized by relatively high water temperatures ($25 \pm 1^\circ\text{C}$), strong luminous intensity ($1605 \pm 335 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high turbidity ($Z_s = 0.7 \pm 0.1 \text{ m}$), alkaline pH (8.26 ± 0.1) and high salinity ($8.6 \pm 2 \text{ ‰}$). Contents in major nutrients, specially nitrogen and phosphorus, are very limiting. Indeed, during the *Microcystis* bloom, soluble reactive phosphorus (P-PO_4) was below the limit of detection, while nitrate concentrations were very low ($0.08 \pm 0.11 \text{ mgN-NO}_3 \cdot \text{L}^{-1}$). The yearly minimal value of the TN/TP atomic ratio (68) coincided with the maximal development of *M. ichthyoblabe*. However, this value remained very high when compared to 20, considered as the maximum limit for the development of cyanobacterial blooms (Paerl 1996). Besides, Smith (1983) reported that cya-

Fig. 2. Temporal variation of chlorophyll a (a) and total phytoplanktonic biomass (b) in the surface of waters during 1999.

Fig. 2. Evolution de la chlorophylle a (a) et de la biomasse phytoplanktonique totale (b) dans les eaux de surface en 1999.

Fig. 3. Seasonal evolution of principal phytoplanktonic classes in the surface of waters during 1999.

Fig. 3. Evolution saisonnière des principaux groupes phytoplanktoniques dans les eaux de surface en 1999.

nobacteria tended to be rare when the atomic ratio TN/TP exceeded 60, from a survey of 17 world-wide lakes. These abiotic conditions are comparable with those quoted for *Microcystis aeruginosa* blooms in Lalla Takerkoust (Sbiyyaa 1997) and El Kansera Moroccan reservoir-lakes (Derraz 1995). The ecological success of *M. ichthyoblabe*, under limiting conditions, depends probably on these ecophysiological properties close to those of many blue-green algae: buoyancy regulation, nutrient storage, phycosphere-microbial associations, high resistance to grazing (Healey 1982, Pettersson et al. 1993, Paerl & Tucker 1995, Paerl 1996).

3.3. *Microcystis ichthyoblabe* bloom toxicity

The toxicity of *M. ichthyoblabe* bloom has been demonstrated by i.p. mouse bioassay. Lyophilised cells sampled in maximal development phase (30/05/1999) give an i.p. LD_{50} of $502 \text{ mg}_{\text{DW}}.\text{kg}^{-1}$ body weight. The autopsy of intoxicated mice reveals that the most affected organ was liver which becomes enlarged and inflated with blood and darker. The mice administrated doses have a low increase in liver weight up to 7.3 % of the total body weight (the control was about 5 %). These symptoms are comparable to those induced by cyanobacterial hepatotoxins.

Fig. 4. Growth kinetic of *Microcystis ichthyoblabe* in the surface waters during 1999.
Fig. 4. Cinétique de croissance de *Microcystis ichthyoblabe* dans les eaux de surface en 1999.

Fig. 5. Vertical distribution of *Microcystis ichthyoblabe* in Oued Mellah reservoir.
Fig. 5. Distribution verticale de *Microcystis ichthyoblabe* dans les eaux du lac-réservoir Oued Mellah.

Photo1. *Microcystis ichthyoblabe* bloom in Oued Mellah reservoir (June 1999).
Photo 1. Bloom à *Microcystis ichthyoblabe* dans le lac-réservoir Oued Mellah (Juin 1999).

Table 3. Some physical and chemical parameters measured in surface waters during and in *M. ichthyoblabe* bloom and no bloom periods in 1999.

Tableau 3. Quelques paramètres physico-chimiques mesurés dans les eaux de surface durant et en dehors de la période du bloom à *M. ichthyoblabe* en 1999.

Parameters	Bloom period (May-June)		Out of bloom	
	min-max	average	min-max	average
Water temperature (°C)	24 – 28	25.6 ± 1.23	12 – 28	19.6 ± 5.9
Incident luminosity ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	1368 – 1842	1605 ± 335	509 – 1912	1266 ± 481
Secchi disk (m)	0.6 – 0.77	0.7 ± 0.1	0.35 – 0.76	0.53 ± 0.16
pH	8.10 – 8.38	8.26 ± 0.08	7.67 – 9.04	8.25 ± 0.42
Conductivity (ms.cm ⁻¹)	3.66 – 4.25	3.99 ± 0.24	2.84 – 4.93	3.94 ± 0.8
Salinity (‰)	7.2 – 10.1	8.64 ± 2.04	6.37 – 12.64	9.35 ± 2.96
SRP ($\mu\text{gP-PO}_4.\text{L}^{-1}$)	0	0	0 – 5.22	0.52 ± 1.65
Nitrates ($\mu\text{gN-NO}_3.\text{L}^{-1}$)	0 – 155	77.5 ± 109.6	66.69 – 2510	854.88 ± 937.5
Atomic TN/TP	68.58 – 195.35	131.96 ± 89.64	92.55 – 421.17	229.51 ± 128.35

In comparison with *M. aeruginosa* bloom of Lalla Takerkoust lake which exhibits a higher toxicity (Sbiyyaa 1997, Oudra et al. 2001), *M. ichthyoblabe* bloom appears to be 6 to 250 times less toxic and could be considered as slightly toxic (LD_{50} between 500 and 1000 $\text{mg}_{\text{DW}}/\text{kg}$) according to Lawton et al. (1994) classification.

3.4. ELISA and HPLC analysis of microcystins

The results of microcystin quantification by ELISA in *M. ichthyoblabe* natural bloom and laboratory isolated strain showed that the total microcystin contents are respectively equal to 0.79 ± 0.25 and 5.4 ± 0.45 $\mu\text{g.g}_{\text{DW}}^{-1}$. The latest values are very low in comparison with the total microcystin contents of 130-650 $\mu\text{g.g}_{\text{DW}}^{-1}$ in *M. aeruginosa* isolated strains and 710-8800 $\mu\text{g.g}_{\text{DW}}^{-1}$ in natural blooms in Lalla Takerkoust lake determined by Oudra (2001). These results explain the low toxicity evaluated by mouse bioassay and agree with the results of Watanabe et al. (1996) which reported that *M. ichthyoblabe* includes both toxic and non-toxic strains.

The presence of microcystins in extracts of both natural bloom and laboratory culture of *M. ichthyoblabe* was confirmed by HPLC-PDA. The HPLC chromatograms (Figs. 6a, 6b) presented the similar peaks with very low absorbances in the case of isolated strain with :

- Absence of the microcystin-LR in comparison with the standard chromatogram (Fig. 6c);

- Presence of 11 peaks corresponding to unidentified microcystins with typical UV spectra. All fractions have higher retention times (RT). This means that these microcystins are more lypophylic (RT between 24.72 and 30.95 min) than LR (RT : 16.24 min), such are LA, LY, LW or LF (Lawton et al. 1994).

The Cyanobacteria blooms in Oued Mellah lake-reservoir as a symptom of increased eutrophication is not only an aesthetic problem, as it reduces recreation, but also an ecological and sanitary problem because of the toxins production by *M. ichthyoblabe* bloom-forming species. In this context, thorough investigations on ecology, physiology, toxicity and toxinology of this

Fig. 6. HPLC chromatograms and UV spectra of *Microcystis ichthyoblabe* bloom (a), isolated strain (b) and microcystin-LR standard (c).

Fig. 6. Chromatogrammes HPLC et spectres UV du bloom à *Microcystis ichthyoblabe* (a), de la souche isolée (b) et du standard de la microcistine-LR (c).

species and other potentially toxic species like *Anabaena aphanizomenoides* and *Prymnesium parvum*, are in progress.

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