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Microcystin ecotypes in a perennial *Planktothrix agardhii* bloom

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ABSTRACT

The dynamics and microcystins (MC) concentrations of a perennial *Planktothrix agardhii* bloom were investigated in a eutrophic lake (Viry-Châtillon, France). A weak relationship was observed between *P. agardhii* population biomass and the MC concentrations in a 1-year survey. To further investigate the causes of MC concentration changes, we concurrently conducted experiments on 41 strains isolated from this lake. We first checked the clonal diversity of *P. agardhii* population (i) by molecular techniques, to assess the presence of MC synthetase gene (*mcy*B), (ii) by biochemical assay (PP2A inhibition assay), for MC production, and (iii) by mass spectrometry (MS), to identify the MC chemotypes. Our results illustrated the diversity of genotype and MC chemotypes within a *P. agardhii* natural population. Eleven chemotypes among the 16 possible ones were found by MS. Furthermore, we noticed major differences in the MC content of isolated strains (from 0.02 to 1.86 μg equiv. MC-LR mg DW^{-1}, $n = 25$). Growth and MC production of one MC-producing strain and one non-MC-producing strain were also assessed at two temperatures (10 and 20 °C). We showed that growth capacities of these strains were similar at the two tested temperatures, and that the MC production rate was correlated to the growth rate for the MC-producing strain. On the basis of these results, several hypotheses are discussed to explain the weakness of relationships between natural *P. agardhii* biomass and MC concentration. One of the main reasons could lie in the proportion of MC-producing clones and non-MC-producing clones that may change during the sampling period. Also, the MC-producing clones may present different intracellular MC content due to (i) MC chemotypes diversity, (ii) changes in MC variants proportions within a strain, and (iii) changes in MC rate production depending on the physiological state of cells. Finally, we concluded that various biological organization levels have to be considered (population, cellular and molecular), through an integrative approach, in order to provide a better understanding of *P. agardhii in situ* MC production.

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1. Introduction

The microbial contamination of surface waters by allochthonous microorganisms, such as enteric bacteria and viruses, or by autochthonous microorganisms may limit the use of water for recreational, agricultural or drinking purposes. These microorganisms include the toxic cyanobacteria occurring naturally in aquatic environments, either trapped in mats or suspended in the water column. The eutrophication of freshwater bodies is now considered as one of the main factors contributing to cyanobacterial blooms formation. These mass occurrences become even more problematic when toxic cyanobacterial blooms occur in recreational areas, or in reservoirs used to provide drinking water.

The filamentous cyanobacterial genus *Planktothrix* is one of the major planktonic cyanobacteria that blooms regularly in Europe (Scheffer et al., 1997; Komarek, 2005). *Planktothrix* blooms are described in a large range of freshwater bodies, including deep alpine lakes (Humbert and Le Berre, 2001) as well as shallow lakes (Berger and Sweers, 1988). *Planktothrix* blooms may be monospecific or associated with other planktonic cyanobacteria, and may occur periodically or persist all year round (Teubner et al., 1999; Berger, 1984; Briand et al., 2002).

Due to their ability to produce microcystins (MC) (for a review see Wiegand and Pflugmacher, 2005), *Planktothrix* blooms are known to pose a significant threat to the health of both animals and humans. Other metabolites (e.g. fatty acids) produced by *Planktothrix* isolates have also been shown to be toxic to fishes and crustaceans (Ernst et al., 2001; Keil et al., 2002). In some cases, *Planktothrix* blooms have been shown to produce more MC per dry weight than *Microcystis* ones (Fastner et al., 1999a; Chorus, 2001).

Planktothrix MC are produced by a large, nonribosomal enzyme complex consisting of peptide synthetases and polyketide synthases. The gene cluster responsible for the biosynthesis of *Planktothrix* MC contains nine genes (*mcy* genes). The arrangements of genes on this cluster, and molecular events such as inactivation, transposition or recombination, directly affect the diversity of MC produced by a strain (Christiansen et al., 2006). As a consequence, the overall toxicity of a *Planktothrix* bloom will depend on both the nature and the diversity of toxins. The toxic potential of *Planktothrix* blooms is also related to (i) the proportion of MC-producing and non-MC-producing sub-populations that make up the population (Kurmayer et al., 2004, 2005; Mbedi et al., 2005) and (ii) the physiological status of the cells (Sivonen, 1990; Wiedner et al., 2003; Tonk et al., 2005; Akcaalan et al., 2006).

Planktothrix agardhii is commonly found in field samples obtained from the Paris area (France). Since 1999, the dynamics and MC production have been studied in a perennial bloom of *P. agardhii* in a shallow eutrophic lake, located near Paris (at Viry-Châtillon). No positive relationship was found between the *P. agardhii* abundance and the concentration of MC (Briand et al., 2002).

In order to investigate this lack of relationship, we investigated this bloom for a further year. Clonal cultures were made from isolated cyanobacterial filaments, and the presence or absence of the *mcy*B gene determined. The overall MC synthesis was investigated by PP2A, and the MC was chemotypes were identified by MS.

The aim of this study was to determine, in a perennial *P. agardhii* bloom (i) the clonal diversity in such a natural population and (ii) the pattern of growth and MC production properties at two temperatures on two isolated clones.

2. Materials and methods

2.1. Environmental sampling

2.1.1. Sampling site

Field samples were taken from the shores of Viry-Châtillon's lake (BNV, Paris area, France; 2°23′04.21″E, 48°40′03.33″N). BNV is a shallow artificial lake originating from a disused sand quarry. It is divided into seven interconnecting reservoirs. The BNV is linked to the river Seine by a channel controlled by a weir. The main hydro-morphological characteristics are described in Briand et al. (2002). BNV has a catchment area of 810 ha. The water residence time in the lake is 0.5 year. Table 1 shows the main physical and chemical characteristics of the lake determined during the survey using the methods described in Briand et al. (2002).

2.1.2. Study of the phytoplankton community

Chlorophyll *a* concentration, dominant cyanobacterial cell abundance and MC concentrations were measured monthly from October 2001 to October 2002.

The chlorophyll *a* concentration was determined spectrophotometrically after methanol extraction (Talling and Driver, 1963). For phytoplankton determination and counting, 10 mL samples of water were fixed immediately after sampling with formaldehyde 5% final (v/v). The microphytoplankton units were counted using a Malassez counting chamber with a Nikon Optiphot 2 microscope ($\times$400, Nikon, Melville, USA). The phytoplankton identification was based on current literature (Komarek and Anagnostidis, 2005). Taxon abundances were determined by counting at least 400 units (cells, trichomes, colonies) to reduce the error to less than 10% ($p = 0.95$; Javornický, 1958). The length of each encountered trichome (*P. agardhii* and *Limnothrix redekei*) has been measured using an ocular micrometer. The cell's widths of these filamentous taxa have been measured for at least 20 trichomes. Biovolumes were then calculated, for each sample, using the formulae described by Sun and Liu (2003).

The MC contents of the field samples were estimated using the protein phosphatase 2A inhibition assay (PP2A) as described in Briand et al. (2002). Under our experimental conditions, the detection limit of the PP2A assay was 10 pg MC-LR. The presence of different MC variants in field samples (February 2002, April 2002, September 2002) was confirmed by mass spectrometry (MS) methods (Robillot and Hennion, 2004).

2.1.3. Sampling, isolation and culture of *P. agardhii* clones from BNV

In March 2001 and from October 2001 to October 2002, samples were collected with a 20-μm-mesh size net in order to isolate *P. agardhii* clones. Water samples were inoculated on

Table 1 – Physical and chemical characteristics of the Viry-Châtillon lake from September 2001 to October 2002

	Mean	Minimum	Maximum	CV (%)
Temperature (°C)	14.3	4.3	24.3	50
PAR (mol $m^{-2} d^{-1}$)	22.1	4.7	41.3	61
pH	8.3	7.9	8.5	2
O_2 concentration ($mg L^{-1}$)	11	9	17	11
Conductivity ($\mu S\,cm^{-1}$)	970	933	1011	2
$N\text{-}NH_4^+$ ($\mu g L^{-1}$)	19	4	38	61
$N\text{-}NO_3^-$ ($\mu g L^{-1}$)	115	0	503	129
$P\text{-}PO_4^{3-}$ ($\mu g L^{-1}$)	20	7	56	77
Total phosphorus ($\mu g L^{-1}$)	453	173	862	50
TSS ($mg L^{-1}$)	25	17	30	17
Secchi depth (m)	0.40	0.34	0.45	10
Chlorophyll *a* ($\mu g L^{-1}$)	47.3	34	68	22

PAR: photosynthetically active radiation; SRP: soluble reactive phosphorus; TSS: total suspended solids; CV: coefficient of variation.

semi-solid Z8 medium (5 g L^{-1} of washed agar, Sigma) (Kotai, 1972). After migrating by phototactism towards a single light source for 24 h, at least 500 individual trichomes per sample were isolated under an inverted microscope. These trichomes were then transferred into Z8 liquid medium in 96-well plates (Rippka, 1988). The plates were placed in growth chambers at 20 ± 1 °C, and illuminated with Cool White fluorescent tubes (Osram Lumilux Plus Eco, L18W/21-840), with a light/dark (L/D) cycle of 16/8 h, under 10–15 μmol photons $m^{-2} s^{-1}$, as measured in empty culture chambers with a LI-250 LICOR quantum meter equipped with a 190SA flat-plate-cosine corrected sensor.

Forty-one growing clones were later cultured in 250-mL Erlenmeyer batch culture flasks containing 100 mL of Z8 medium and incubated under the same conditions as used during the isolation step. These clone cultures are maintained in the Paris Museum Collection (PMC).

2.2. *Characterization of the mcyB genes, toxicity and MC chemotypes of the P. agardhii clones*

2.2.1. *Amplification of 16S rDNA and of the mcyB genes*

The primers CYA 106F (Nübel et al., 1997) and Pk2R (5′-TGGTTCCTTACGGCACTCT-3′) were chosen for 16S rDNA amplification of the *Planktothrix* genus. The gene *mcy*B was selected for MC gene amplification as it displays limited sequence variability in the *Planktothrix* genus (Kurmayer et al., 2005; Mbedi et al., 2005). Two primers, specifically designed to amplify the first adenylation domain of the *mcy*B gene, were kindly provided by G. Christiansen (A4F: 5′-AGCTATCCTAC-GACAAACCGC; A6R: 5′-TCAGCCGTTAACTCAAGCTGA). Reactions were performed in 25 μL, containing 1 μL of clonal culture, 200 μM of dNTP, 10 μM of each primer and 5 μl 10 × PCR buffer. The template was frozen at −20 °C, and then boiled three times for 1 min before adding 1 U Taq polymerase (Eppendorf, Germany). The thermal-cycling conditions were as follows: 5 min denaturing at 94 °C; 38–30 s cycles denaturing at 94 °C, 30 s annealing at 60 °C, and 30 s extension at 72 °C, followed by 5 min elongation at 72 °C. The amplified products were checked on 1.5% agarose gels and visualized by ethidium bromide staining.

The two sets of primers, specific of the 16S rDNA gene and the *mcy*B gene of *Planktothrix*, were combined into a multiplex PCR. The products were then sequenced using the same *Planktothrix* *mcy*B primer set as used for the amplification. DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) apparatus. The nucleotide sequences obtained in this study have been deposited in the EMBL database under the following accession numbers: partial 16S rDNA of *P. agardhii* PMC 75.02, AJ544065; *P. agardhii* PMC 87.02, AJ544066; and partial *mcy*B gene of *P. agardhii* PMC 75.02, AJ544071.

2.2.2. *MC synthesis*

The 41 *P. agardhii* strain cultures were screened for MC concentration using the PP2A inhibition bioassay. Each culture was monitored by optical density at 750 nm ($OD_{750\,nm}$, Cary 50 Varian spectrophotometer) (Post et al., 1985) and was stopped in the late exponential phase ($OD_{750\,nm} \approx 0.45$–$0.50$). About 20 mL of each culture was used for PP2a analysis corresponding to a total cellular biovolume between 12 and 13 mm^3.

2.2.3. *MC chemotypes*

For each strain, the MC variant composition was studied by MS. Mass spectra data were performed with a typical time-of-flight (Tof) hybrid tandem mass spectrometer (Q-Star Pulsar i Applied Biosystems) equipped with an electrospray ionization (ESI) source. All experiments were performed in positive-ion mode. Data were acquired and processed using Analyst Qs software (Applied Biosystems, Framingham, MA, USA). The capillary voltage was set to 2500 V, and the declustering potential was typically 20 V for the Tof-MS mode. The mass scan range was from *m/z* 800 to 1200, and the scan cycle was 1 s. For MS/MS experiments, the mass scan ranged from *m/z* 30 to 170 (to select the ion at *m/z* 136), and the collision energy was set to 30 eV with a declustering potential of 60 V. The detection limit of MC-LR by the described MS method was 1 ng.

2.2.4. *Growth and toxicity variations*

The growth rates of an MC-producing strain (PMC 75.02) and a non-MC-producing (PMC 87.02) strain were compared at two temperatures (10 and 20 °C).

Pre-cultures of the two strains were used to inoculate 250-mL Pyrex Erlenmeyer flasks containing 90 mL of Z8 sterile medium. All the experiments were performed in triplicate. The inoculums used had an $OD_{750\,nm}$ of 0.07. After inoculation, the flasks were placed in identical growth chambers at 10 and 20 °C. Each day, the cultures were shaken by hand to ensure that the flask contents were mixed. They were moved randomly with respect to the light source in order to increase the light regime homogeneity ($24 \pm 4\,\mu mol$ photons $m^{-2}\,s^{-1}$ with a 16/8 L/D photoperiod). The growth kinetics was monitored by $OD_{750\,nm}$, dry weight, and chlorophyll *a* concentration, for 34 days at 20 °C and 65 days at 10 °C. Depending on the cell density, 10–50 mL of culture was then filtered over 47 mm GF/C (Whatman) for chlorophyll *a*, dry weight and toxin analysis. Chlorophyll *a* concentration was determined as described above. The dry weight was measured after a 24 h drying at 105 °C. The maximum growth rate (μ_{max}) was calculated from the data provided by OD_{750}, chlorophyll *a* and dry weight as described by Guillard (1973). The similarity of the μ_{max} was tested by covariance analysis (ANCOVA) according to Zar's method (Zar, 1999) with GraphPad Prism v.4 software (Graph Pad Inc., San Diego, USA).

The overall MC cell content during the growth of the MC-producing strain was evaluated by PP2A bioassay.

3. Results

3.1. BNV study

3.1.1. Phytoplankton and MC dynamics

During the survey, BNV was characterized as eutrophic on the basis of the chlorophyll *a* and Secchi disk values (mean [chl *a*] = 47 $\mu g\,L^{-1}$; mean Secchi disk = 0.4 m; Table 1). Chlorophyll *a* concentration ranged from a maximum value of 68.3 $\mu g\,L^{-1}$ in October 2001 to a minimum value of 34 $\mu g\,L^{-1}$ recorded in May 2002 (Fig. 1).

P. agardhii was the dominant cyanobacterium (>90% of total phytoplanktonic counts) throughout the year, with a maximum biovolume of 605 $mm^3\,L^{-1}$ in October 2001 and a minimum biovolume of 82 $mm^3\,L^{-1}$ in June 2002 (Fig. 1). Except for a few other sporadically detected species, only trichomes of *L. redekei* formed significantly countable biomass. However, *L. redekei* represented always less than 10% of the total phytoplankton biovolume.

The highest MC concentration (34.5 μg equiv. MC-LR L^{-1}) was observed in October 2001 (Fig. 1), and the lowest (2 μg equiv. MC-LR L^{-1}) in July and August 2002. Four MC variants (981.5, 1024.5, 1031.7 and 1045.5 *m/z*) were detected by MS on three natural samples (February, April and September 2002).

The relationship between MC concentrations and *Planktothrix* biovolume is illustrated in Fig. 2. When a linear regression model was fitted to the observed data, a significant positive relationship was observed ($R^2 = 0.587$, $p < 0.01$). However, the significance of the relationship was weak because it was mainly supported by a single value (October 2001) corresponding to both the maxima of *P. agardhii* biovolume and MC concentration. If this value is not added to the regression model, the relationship between biomass and MC concentration in the field samples is no longer significant ($R^2 = 0.103$, $p > 0.05$).

3.2. Characterization of the P. agardhii strains isolated

3.2.1. Strain isolation

The efficiency of clone isolation varied from month to month and ranged from 0% to 4% (mean = 1.2%). Eighty-six non-axenic clonal strains of *Planktothrix* gave positive growth results under our laboratory conditions (7100 trichomes were initially isolated). Forty-one of these 86 strains were conserved in the PMC collection (Table 2), and examined further in this study.

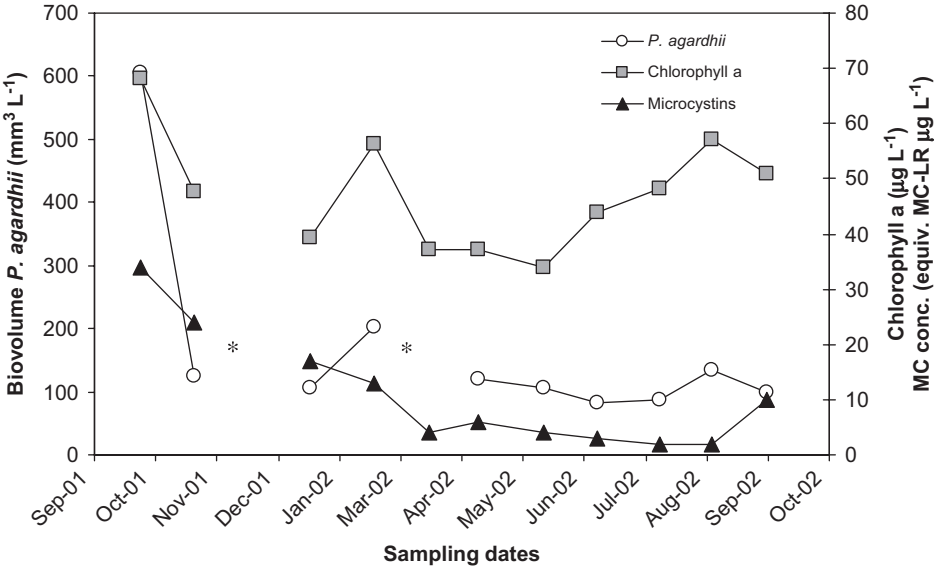

Fig. 1 – Dynamics of *Planktothrix agardhii* biovolume (in $mm^3\,L^{-1}$), phytoplankton biomass (in μg chlorophyll $a\,L^{-1}$) and MC concentration (in μg equiv. MC-LR L^{-1}) in BNV from September 2001 to October 2002. *, not done.

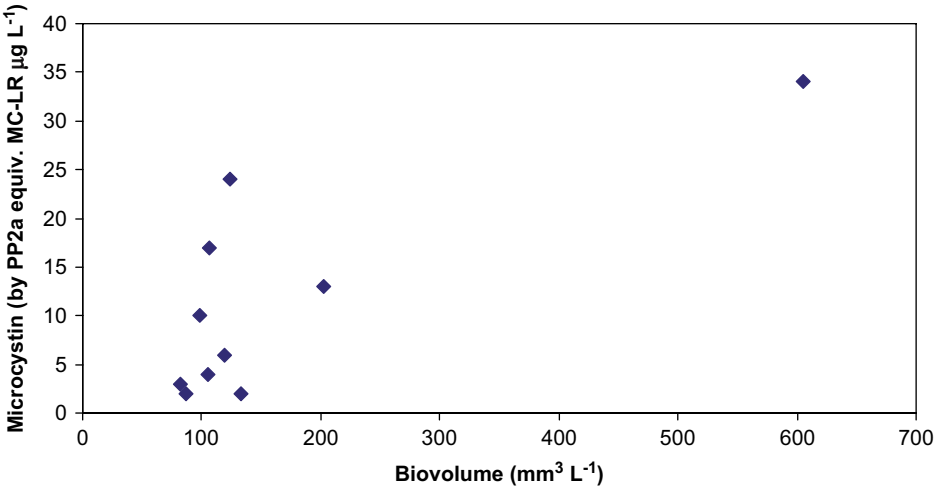

Fig. 2 – MC concentration (in µg equiv. MC-LR L^{-1}) versus *Planktothrix* biovolume (in $mm^3 L^{-1}$) in BNV from September 2001 to October 2002.

Table 2 – MC chemotypes detected among the 41 strains of *P. agardhii* isolated from the BNV lake

MC chemotypes (*m/z*)	Number of strains (*n*) and PMC number	*mcyB*	PP2a	MC content (µg equiv. MC-LR mg Dw^{-1})			
				Min.	Max.	Mean	SD
981.4, 1024.5, 1045.4	(9): 80.02, 81.02, 120.02, 121.02, 105.02, 108.02, 109.02, 172.02, 213.02	+	+	0.12	1.44	0.6 ($n = 3$)	0.7
981.4, 1045.4	(8): 73.02, 75.02, 76.02, 77.02, 78.02, 79.02, 100.02, 180.02	+	+	0.34	1,86	1.0 ($n = 6$)	0.5
981.4	(4): 51.01, 90.02, 119.02, 123.02	+	+	0.02	0.46	0.3 ($n = 3$)	0.2
981.4, 1024.5, 1030.7	(4): 74.02, 82.02, 86.02, 102.02	+	+	0.17	0.95	0.6 ($n = 3$)	0.4
981.4, 1024.5	(3): 98.02, 107.02, 212.03	+	+				
981.4, 1030.7, 1045.4	(2): 52.01, 72.02	+	+				
1024.5	(1): 88.02,	+	+				
1030.7	(2): 92.02, 96.02	+	+				
981.4, 1024.5, 1030.7, 1045.4	(1): 91.02	+	+				
1024.5, 1030.7	(1): 103.02	+	+				
981.4, 1030.7	(0)	–	–				
1045.4	(0)	–	–				
1030.7, 1045.4	(0)	–	–				
1024.5, 1045.4	(0)	–	–				
1024.5, 1030.7, 1045.4	(0)	–	–				
No variant	(6): 50.01, 87.02, 122.02, 101.02, 179.02, (104.02)	–(+)	–(+)				

Each strain, represented by a PMC collection number, was screened for the presence (+) or absence (–) of the *mcy*B gene, the response (+ or –) to PP2a assay and the MC concentration (only reported when the number of MC concentration and dry weight simultaneous estimations was ⩾3).

3.2.2. *MC genotypes and chemotypes*

The multiplex PCR test used for *Planktothrix* clonal cultures allowed one to distinguish between MC-producing and non-MC-producing clones. Two products, of 902 and 554 bp, corresponding to the 16S rDNA and *mcy*B genes, respectively, were amplified in the MC-producing clones. Only the 16S rDNA gene amplification product could be detected for the non-MC-producing clones. The partial 554-bp *mcy*B sequence from PMC 75.02 was similar (98% identity) to the gene of *P. agardhii* CYA126 (AJ441056; Christiansen et al., 2003). Of the 11 differing nucleotides, seven were silent changes that did not modify the protein sequence. The other four consisted in changes on the first and second codons varying the *mcy*B protein sequence. As the *mcy*B is responsible for the activation of leucine, arginine or homotyrosine residues in position 2, these changes in amino acid sequence from PMC 75.02 induce modifications in the MC final structure (Kurmayer et al., 2005). The main difference between the two strains in MC-variant composition is [Asp^3, MC-HtyR] for PMC 75.02 compared to [Asp^3, $Mdha^7$-MC-RR] for CYA126.

The 41 *P. agardhii* strains were first screened for the presence/absence of one of the genes necessary for MC biosynthesis (*mcy*B), then for their MC-producing potential (by PP2A inhibition assay), and finally for their MC variants profile by MS (Table 2).

Of the 41 strains screened, 36 were *mcy*B positive (*mcy*B$^+$) and five *mcy*B negative (*mcy*B$^-$) (Table 2). The 36 *mcy*B$^+$ strains displayed PP2A inhibition. To identify MC variants, the masses of all peaks between 800 and 1200 were compared to known MC masses found in the literature, and confirmed by identifying the ADDA fragment mass by MS–MS. By doing so, four variants were detected (Table 2).

Three categories of strains can be distinguished: (1) a most often encountered one ($n = 35$), formed by the *mcy*B$^+$, PP2A$^+$ and MC$^+$ strains, which were classified as MC-producing strains; (2) a second category consisting of non-MC-producing strains ($n = 5$), which were *mcy*B$^-$, PP2A$^-$ and MC$^-$; (3), and a third category composed of a single strain (PMC 104.02), which was *mcy*B$^+$, PP2A$^+$, but for which no MC could be detected by MS.

Eleven of the 16 possible different chemotypes were characterized in the MC-producing strains (Table 2). Seven strains produced a single MC variant, whereas 12 strains produced two variants and 15 strains were able to produce three variants. Only one strain (PMC 91.02) produced all four different MC variants (Table 2).

Two toxin profiles (981.5, 1024.5, 1045.5 *m/z* and 981.5, 1045.5 *m/z*) accounted for 41% of the strains ($n = 17$, Table 2).

Analysis of the MC variants produced by the 41 strains screened showed that the most often encountered variant was 981.5 *m/z*, followed by 1045.5, 1024.5 *m/z* and then 1031.7 *m/z* (Table 2).

The range of MC contents of the four most common chemotypes did not differ significantly. But, considering the MC content of all the strains estimated by PP2A assay, it ranged from 0.02 to 1.86 μg equiv. MC-LR mg DW^{-1} (Table 2).

3.2.3. *In vitro variations of growth rate and MC production*

The growth rate was recorded at two temperatures (10 and 20 °C) for one MC-producing strain (PMC 75.02) and one non-MC-producing strain (PMC 87.02) (Table 3). Three proxies (OD, chlorophyll *a* and dry weight) were used to measure changes in *P. agardhii* clonal culture biomass. The correlation coefficients were closely related to each other in the exponential phase (OD_{750}/Chl *a*, $R^2 = 0.949$, $p < 10^{-4}$; OD_{750}/DW, $R^2 = 0.955$, $p < 10^{-4}$; DW/Chl *a*, $R^2 = 0.911$, $p < 10^{-4}$).

The growth rates of the two strains were not significantly different at the two temperatures tested (Table 3). Both strains displayed faster growth at 20 °C, but growth remained high at 10 °C ($\mu \approx 0.1\,d^{-1}$). The stationary phase was reached later at 10 °C than at 20 °C. Consequently, an unexpected higher final biomass was reached at 10 °C.

MC production was also studied at both temperatures for the MC-producing strain (PMC 75.02). During the exponential phase, there was a highly significant linear relationship between the dry weight and the MC concentrations at both the temperatures tested in culture ($R^2 = 0.973$ at 10 °C and $R^2 = 0.919$ at 20 °C; Fig. 3).

The slopes relating MC concentrations and biomass were not significantly different (ANCOVA, $p > 0.696$) at the two temperatures tested (Fig. 3); this was also confirmed when the data were pooled together ($y = 1.7945 \times -3.783$; $R^2 = 0.951$; $p < 10^{-4}$). The slope of the regression curves indicated that MC concentrations (μg equiv. MC-LR L^{-1}) increased faster than biomass (mg dry weight L^{-1}).

4. Discussion

4.1. *Dominance of P. agardhii in BNV*

The year-round dominance of *P. agardhii* in the BNV water body was already reported in a previous survey (Briand et al., 2002), suggesting that this species was particularly well adapted to this environment. The other species observed, *L. redekei*, never exceeded 10% of the total phytoplanktonic biomass. These two species belong to the same functional group as described by Reynolds et al. (2002) (S1 group), typical of enriched shallow lakes where monospecific population can persist throughout the year. Generally, in the temperate lakes of the northern hemisphere, *P. agardhii* frequently dominates the phytoplankton community of such eutrophic water bodies (Feuillade, 1994; Nixdorf et al., 2003; Briand et al., 2005).

P. agardhii was dominant all year round, but it exhibited changes in biomass, with minimal and maximal values recorded during early and late summer, respectively. These periods do not coincide with those reported by Briand et al. (2002), indicating that the dynamics of this *P. agardhii* perennial population is variable, and does not display any

Table 3 – Growth rate (μ) and population doubling time of two strains of *P. agardhii* (PMC 75.02 (MC-producing) and *P. agardhii* PMC 87.02 (non-MC-producing)) were calculated in the exponential phase growth

	PMC 75.02 (MC$^+$)		PMC 87.02 (MC$^-$)	
	20 °C	10 °C	20 °C	10 °C
μ (d^{-1})	0.162 ± 0.019	0.083 ± 0.01	0.159 ± 0.048	0.099 ± 0.019
Doubling time (d)	4.3	8.3	4.4	7
Stationary phase (d)	12	≈50	16	≈50
Dry weight (mg L^{-1}) maximal value	350	≈734	283	≈548

Two temperatures were tested, 10 and 20 °C. ±: confidence interval at the 0.05 level.

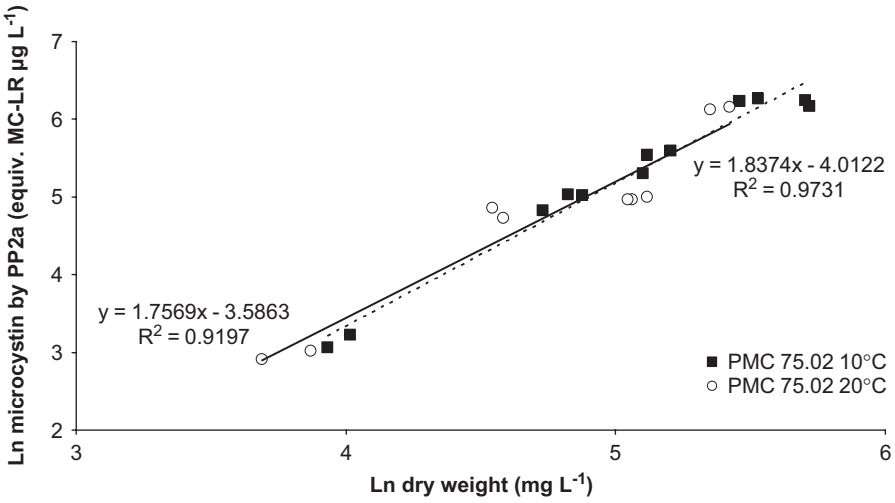

Fig. 3 – Relationship between MC concentration and dry weight during exponential growth of the MC-producing *P. agardhii* strain PMC 75.02 at 10 and 20 °C. The slopes and origin intercepts of the two linear regressions are not significatively different (ANCOVA, $p > 0.696$).

clear seasonal cycle. This suggests a multifactorial control not directly related to seasonal climate changes.

4.2. *Genotypic and chemotypic diversity of P. agardhii strains from BNV*

Few studies have been published about the growth characteristics of MC-producing and non-MC-producing strains within the population of a single water body. It was particularly interesting to investigate this in the BNV lake, due to the presence of a perennial bloom and the weakness of the relationship between biomass and MC concentration. In their recent study, Rantala et al. (2006) revealed, using genus-specific MC synthase *mcy*E PCR, that 63% of the Finnish lake phytoplankton communities they studied contained potentially MC-producing *P. agardhii* clones. Mbedi et al. (2005) tested 17 *P. agardhii* strains isolated from Lake Maxsee and seven from River Erdre, and found that MC-producing strains accounted for 41% and 0% of the resident populations, respectively. Our study illustrated possible MC-chemotype variability within natural populations of *P. agardhii*. But it was impossible to infer the extent of this variability, because of the difficulty of isolating trichomes and culturing them. Several hundred *P. agardhii* trichomes were isolated each month and then cultured. There was little likelihood of obtaining a clonal culture from an isolated trichome (1.2%). This low efficiency could be explained by the isolation steps (based on selecting mobile trichomes) and/or culture conditions. However, this could not explain the differences in the rate of culture success from one month to another (for example, in June and September 2002 no positive growth was obtained; data not shown). The success of the isolation/culture steps could be checked from the physiological state of the *Planktothrix* trichomes when sampled and isolated. Our results differed from those published by Messineo et al. (2006), who successfully obtained 100% growth from 40 single *P. rubescens* organisms isolated from natural water samples. Low culture success is a well-known feature of aquatic heterotrophic bacteria, and new methods have been proposed to improve the proportion of cells that can be cultured, and to access further characterization of bacterial species from natural communities (e.g. Zengler et al., 2002; Selje et al., 2005).

Among the 41 clones screened, two categories were expected: MC-producing (*mcy*B$^+$, PP2A$^+$, MC$^+$) and non-MC-producing (*mcy*B$^-$, PP2A$^-$, MC$^-$), corresponding to 85% and 12% of the cultured strains, respectively. The third category consisted of a single strain (PMC 104.02), and was surprisingly both *mcy*B$^+$ and PP2a$^+$, but no MC variants were detected by MS. We can hypothesize that the MC synthesis rate of this particular strain may be so low that variants could not be detected by MS; under our experimental conditions, the PP2A bioassay was 100 times more sensitive than MS. Such discrepancies between protein phosphatase inhibition assay and MS had already been observed by Kurmayer et al. (2004).

Four MC variants (from strains and field samples) were identified on the basis of their molecular mass and fragmentation profile. Five MC variants had previously been identified in BNV by HPLC-ES-MS (Briand et al., 2002; Robillot and Hennion, 2004): [D-Asp^3]MC-LR (981.5 *m/z*), [D-Asp^3]MC-RR (1024.5 *m/z*), MC-RR (1038 *m/z*), MC-YR (1045.5 *m/z*), plus the one at 1031.7 *m/z*. As MC variants produced either by *Planktothrix* natural samples or by isolates consist mainly of the demethylated forms (Fastner et al., 1999b; Kurmayer et al., 2004; Welker et al., 2004), we hypothesized that MC-981.5 *m/z* corresponds to [D-Asp^3]MC-LR, MC-1024.5 *m/z* to [D-Asp^3]MC-RR and MC-1045.5 *m/z* to [D-Asp^3]MC-HtyR; the MC-1031.7 *m/z* remains to be determined. One of the most important differences between these two surveys (that of Briand et al. (2002) and the present study) is the fact that MC-RR (1038 *m/z*) was not observed in either the field samples or the strains.

Eleven of the 16 possible chemotypes were observed among the MC-producing strains with four different MC variants. It has already been demonstrated that each variant displays a

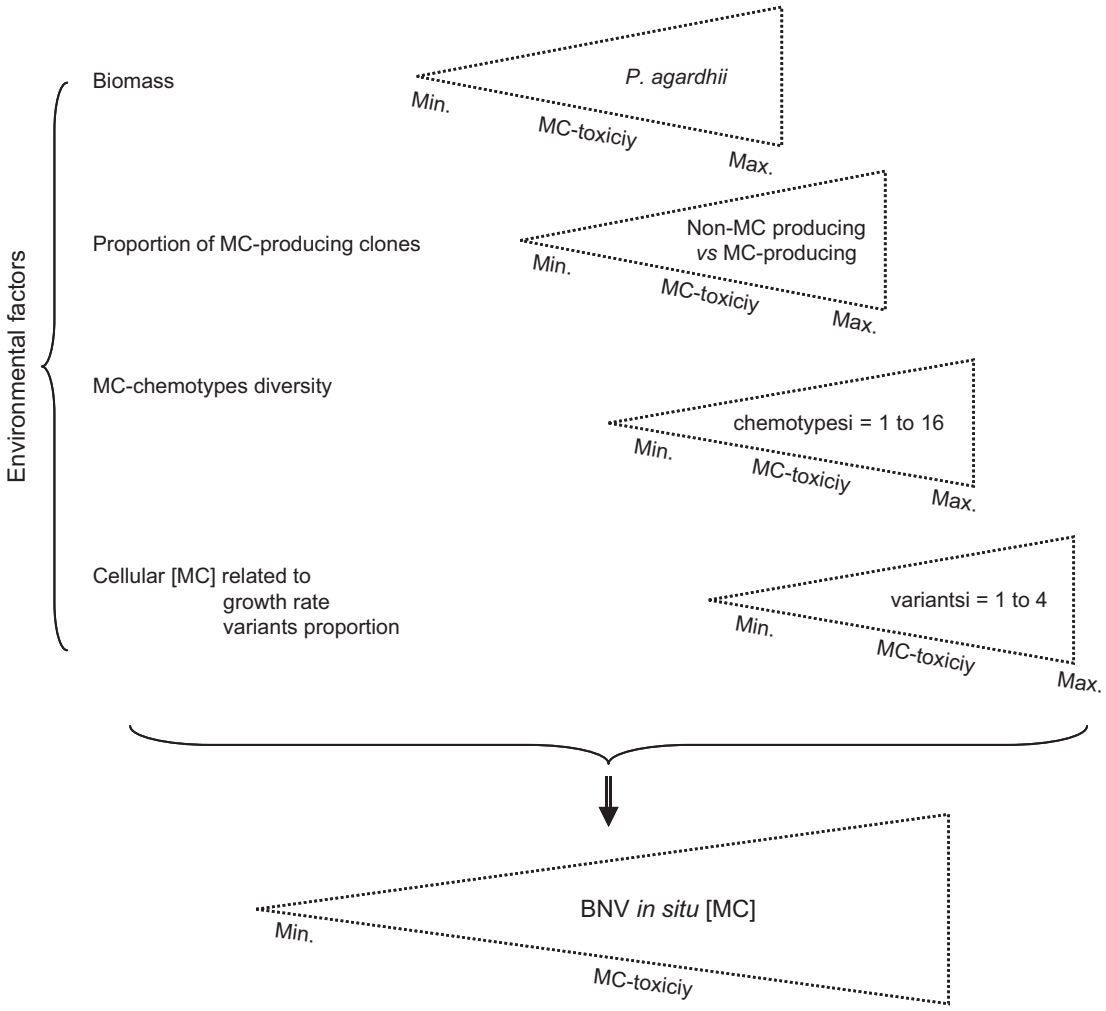

Fig. 4 – Conceptual diagram showing the different biological levels that can affect the MC concentration in the BNV water body.

different level of toxicity (e.g. on mouse or *Thamnocephalus platyurus*), and this allows us to hypothesize that each MC combination may induce a different degree of toxicity (Sivonen and Jones, 1999; Blom et al., 2001). This diversity of chemotypes could also result in variability in the PP2A test, due to the number and nature of the MC that a strain is able to synthesize (Robillot and Hennion, 2004; Blom and Juttner, 2005). This could have introduced some degree of bias into our evaluation of the MC concentration (*in situ* and on strains), but could not be sufficient enough to explain the major differences that were noticed in the amount of MCs per biomass unit for the different strains (e.g. near $\times 90$ between 0.02 and 1.86 μg equiv. MC-LR per mg of dry weight, Table 2). Such differences between the MC contents of different strains were greater than those found for the set of strains tested by Kurmayer et al. (2005), and conflict with the hypothesis suggesting that all the MC-producing strains may have a cellular MC content within a fairly narrow range (Briand et al., 2005). These differences from one strain to another may explain why such a large range of MC concentrations can be observed for the same *Planktothrix* biomass in a natural population.

The presence of the *mcy*B gene does not necessarily imply MC toxicity or MC synthesis (Kurmayer et al., 2005; Gobler et al., 2007; Christiansen et al., 2006). The MC gene can be inactivated as a result of recombination, insertion or deletion events, which are frequently observed in cyanobacteria (Mazel et al., 1991; Kurmayer and Gumberger, 2006). Kurmayer and Gumpenberger demonstrated that in *Planktothrix* strains genetic variations in *mcy*BA1 sequences can induce amino-acid variability in position 2 of the MC molecule (e.g. Arg or Hty/Leu or Arg/Leu or Arg/Hty/Leu). The recombination events in *P. agardhii* populations frequently appear to induce genetic heterogeneity. This may be a reason for the diversity of MC genotypes and chemotypes observed within the BNV *P. agardhii* population.

Furthermore, several authors have reported transcriptional regulation at a molecular level. For example, Tonk et al. (2005) have demonstrated that light intensity can influence the proportions of variants within a given strain of *P. agardhii*. Transcription can also be modified by nitrogen concentration or iron, as suggested by Martin-Luna et al. (2006). Such observations indicate that environmental factors influence MC synthesis at a cellular level.

Thus, for a given *P. agardhii* population the intrinsic capacity to produce toxins appeared to be quite diverse from both a qualitative and quantitative point of view.

4.3. MC concentration dynamics and related hypothesis in BNV

The MC concentration dynamics in BNV is probably under multifactorial control as illustrated in Fig. 4. Considering the weak relationships between the *P. agardhii* biomass and the *in situ* MC concentration in BNV (this study, Briand et al., 2002), two main hypotheses can be discussed:

(1) *Varying abundance of MC-producing and non-MC-producing clones*: Such variations have already been reported within natural populations of *Planktothrix* (Kurmayer et al., 2004; Mbedi et al., 2005). The varying abundance of MC-producing and non-MC-producing sub-populations in the environment can be linked to the differing growth performances of the two sub-populations. In this study, growth experiments on an MC-producing strain (PMC 75.02) and a non-MC-producing strain (PMC 87.02) showed similar patterns of growth when cultured at the two tested temperatures. This could indicate that the emergence of dominant MC-producing strains in the ecosystem may not be linked to better growth capacities. However, our results do not exclude the possibility that growth of these two types of strains could be mediated by environmental parameters other than temperature such as competition in a natural multiclonal population.
(2) *Variable intracellular MC content*: As revealed by Gobler et al. (2007) during a *Microcystis* survey in a New York lake, the expression of the MC synthetase gene is variable. Furthermore, intracellular MC concentration is influenced by the physiological state as shown for *Planktothrix rubescens* (Briand et al., 2005) and for *Microcystis* (Downing et al., 2005). It has been demonstrated that MC production by *P. rubescens* is related to positive growth, and that the intracellular concentration of MC can vary during the different phases of growth. This hypothesis is confirmed in the present study by the *in vitro* experiments conducted on the MC-producing strain (PMC 75.02) isolated from BNV (Fig. 3). There was a highly significant relationship ($R^2 = 0.951$) between growth and MC synthesis during the exponential phase, but not from the stationary phase to the end of the culture. This observation could be one of the explanations of the lack of a strong relationship between biomass and *in situ* MC concentrations.

5. Conclusion

This study is the first report of genotypic and chemotypic diversity within a monospecific population of *P. agardhii* isolated from a single water body. Such infra-specific diversity needs to be considered when trying to identify the causes of MC concentration changes within a *P. agardhii* bloom, such as the one observed in the BNV. Moreover, these results call for further experimentations to elucidate (i) the role of environmental factors on the different biological regulation levels of MC cellular concentration, (ii) the consequences of infra-specific diversity in terms of adaptative capacities of *P. agardhii*, and (iii) the relative importance of the different regulation levels that are implied in the MC *in situ* concentration. Regarding this last point, in a perennial bloom where biomass dynamics is limited, the other regulation levels (e.g. proportion of MC-producing clones) may have a significant role in MC concentration changes.

Thus, the evaluation of toxicity and related public health consequences of cyanobacterial perennial blooms need not only be a survey of phytoplankton biomass, but also of the associated toxin concentrations.

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