Microbial Ecology

Variations in the Microcystin Production of *Planktothrix rubescens* (Cyanobacteria) Assessed from a Four-Year Survey of Lac du Bourget (France) and from Laboratory Experiments

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Abstract

Between 1999 and 2002, a routine survey of water quality in the Lac du Bourget was performed to study the dynamics and microcystin (MC) production of *Planktothrix rubescens*. Using liquid chromatography coupled to diode array detection and mass spectrometry, we found that two main variants ([D-Asp^3] and [D-Asp^3, Dhb^7] microcystin-RR) were produced. The proportion of these two variants was not influenced by the depth or season of sampling. Expressed in microcystin-LR equivalents, high microcystin concentrations were recorded from August to December each year, reaching values of up to 6.7 μg L^{-1}. A significant correlation was found between the microcystin cell content and the cell densities of *P. rubescens*. Cellular quotas of microcystins ranged from 0.1 to 0.3 pg $cell^{-1}$. Simultaneously, laboratory experiments were performed on a strain of *P. rubescens* isolated from the lake to assess the potential impact of various P–PO_4^{3-} concentrations on intra- and extracellular microcystin production. Unlike natural populations, this strain only produced [D-Asp^3] MC-RR. The intracellular microcystin content was similarly correlated to the cell density, but the cellular quota was slightly higher (0.3–0.7 pg $cell^{-1}$) than in the natural population. Again, as in the natural population, a linear relationship was found between growth rate and microcystin production rate. These findings support the hypothesis that environmental factors, such as phosphate concentrations, have no direct impact on microcystin production by *P. rubescens*, but act indirectly by affecting growth rate.

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Introduction

In recent years, an increasing number of papers have been published that deal with problems arising from proliferations of toxic cyanobacteria in aquatic ecosystems. These proliferations induce ecological disturbances such as a decrease in aquatic biodiversity, with potential consequences for the entire trophic web [23, 30]. The toxic potential of several cyanobacterial genera may also represent considerable health risks for both wild and domestic animals (e.g., [4]) as well as human beings [22]. So far, most of the studies dealing with cyanotoxin production have mainly concerned either *Microcystis aeruginosa* (e.g., [15, 18, 46]) or *Planktothrix agardhii* (e.g., [5, 47]) because these are the two most prevalent species in eutrophic freshwater ecosystems.

It is noteworthy, however, that cyanobacterial toxins can also affect marine and brackish ecosystems (*Lyngbya majuscula* and *Nodularia spumigena*, respectively) [39], polar ponds (benthic mats of *Oscillatoriales*) [12], and hot African springs (*Synechococcus* spp. and *Oscillatoriales* species) [19]. Toxic cyanobacteria can also occur in oligotrophic alpine lakes (benthic *Oscillatoria* spp.) [28] and in deep mesotrophic subalpine lakes. In the latter case, *Planktothrix rubescens* is currently the dominant species reported in the following lakes: Zurich (Switzerland) [44], Garda (Italy) [36], Mondsee (Austria) [21], Geneva (France and Switzerland) [1], Nantua (France)

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[10], and Bourget (France) [14]. In Lac du Bourget, the proliferation of *P. rubescens* has been associated with the process of restoring this ecosystem [16]. Indeed, it has been suggested that in this ecosystem, blooms may be linked to increasing transparency (because of a decrease in phytoplankton biomass linked to the lower phosphorus concentrations) in the near-surface layer and to a longer stratified period (because of global warming).

To date, the toxicity of *P. rubescens* has always been attributed to the hepatotoxic microcystins [2, 8, 9, 14]. Microcystin-producing cyanobacteria generally synthesize between 4 and 10 different variant microcystins, whereas *P. rubescens* exhibits a simpler toxin profile consisting of two to four different microcystins [9, 26]. Amino acid and nuclear magnetic resonance (NMR) analyses, coupled with mass spectrometry (MS), have identified demethyl variants of microcystin-RR ([D-Asp^3, Dhb^7] MC-RR, [D-Asp^3] MC-RR, or [Dha^7] MC-RR) [2, 9, 26]. The closely related species, *P. agardhii* (see [13]), displays the same pattern, whereas *Microcystis* spp. mainly produces nondemethylated variants [9].

Data are still scarce about the effect of environmental parameters on the production of cyanotoxins. To the best of our knowledge, field and laboratory investigations have been confined to microcystins (MC), apart for two studies involving anatoxin-a [33] and saxitoxins [7]. In addition, these reports have mainly concerned *Microcystis* spp., apart from two studies of *P. agardhii* [3, 38] and one of *Anabaena* spp. [34]. Under experimental conditions, attempts to demonstrate the impact on microcystin production/regulation of various factors such as the levels of phosphorus [38, 45] and nitrogen [25, 31, 38], temperature [34, 38, 45], light [38, 41, 48], pH [17, 40], and iron [42] have led to contradictory conclusions. However, it does look as if the concentrations of phosphorus and/or nitrogen [11, 25, 29, 31] as well as the light level [41, 48] more probably act indirectly via an influence on growth rates.

Thus, the question of how environmental factors influence the toxicity of cyanobacteria remains an important challenge facing researchers. To contribute to this debate, we studied the influence of environmental parameters on the dynamics and toxicity of *P. rubescens* by means of both a field survey in Lac du Bourget and laboratory experiments using batch cultures of a *P. rubescens* strain isolated from the lake and stressed by exposure to various concentrations of phosphates.

Materials and Methods

The Lake Studied. Lac du Bourget (45°44′N, 05°51′W, 231-m altitude) is the largest natural French lake and is located in the eastern part of France, at the foot of the Alps. It is a meromictic lake, is elongated in shape (18 and 3 km in length and width, respectively), and north–south orientated. It has a surface area of 42×10^6 m^2, a total volume of 3.5×10^9 m^3, maximum and average depths of 145 and 80 m, respectively, and a water residence time of approximately 10 years. Winter overturn reaches the bottom of the lake only during very cold winters. Two rivers, the Leysse and the Sierroz, are the main freshwater inputs to the lake, with average flow rates of 8.5 and 3.5 m^3 s^{-1}, respectively. There are two main cities on the banks of the lake, Chambéry in the south and Aix-les-Bains in the east, with a total of approximately 170,000–200,000 inhabitants. The lake is a major resource used for drinking water, fisheries, and recreational activities.

Water Sampling and Cell Counting. Water samples were collected at reference station "B" (corresponding to the deepest part of the lake, i.e., 145 m) [16] from July 1999 to January 2003. Samples were taken once or twice a month from seven different depths (2, 6, 10, 15, 20, 30, and 50 m) and used for cyanobacteria counts. Preserved in Lugol's iodine solution was 0.3 L of each sample. Two-hundred-micrometer units of *P. rubescens* filaments were counted using the Utermöhl inverted microscope technique after sedimenting 50 mL water. Estimation of the number of cells was obtained knowing that the mean length of a *P. rubescens* cell (estimated on 100 measurements) was 5 μm. For microcystin analyses, 1-L samples of water were collected at the same depths, but systematic analyses were only performed at the 10-, 15-, and 20-m depths, corresponding to the layer where the *P. rubescens* biomass is highest [16].

Laboratory Cultures. For the laboratory experiments, we used the monoclonal but nonaxenic strain *P. rubescens* TCC 29 (Thonon Culture Collection ref 29), which was isolated from Lac du Bourget in September 2001. A preculture of this strain was grown under continuous conditions in a 5-L turbidostat-like system supplied with Z medium [6] at 15°C and under 20 μmol photons m^{-2} s^{-1} provided by daylight fluorescent tubes (OSRAM Lumilux® "de luxe"), with a 12:12 h light/dark cycle. Inoculates of this preculture were used to prepare batch cultures in autoclaved 250-mL Pyrex Erlenmeyer flasks containing 100 mL of Z medium to test the effect of P–PO_4 on the growth and the microcystin production of *P. rubescens*. A total of 75 Erlenmeyer flasks were inoculated at five P–PO_4 concentrations (final concentrations of 0.005, 0.02, 0.06, 0.34, and 5.5 mg/L P–PO_4, referred to as Z0, Z1, Z2, Z3, and Z respectively) under the same temperature and light conditions as the preculture. These conditions were selected to reproduce the different concentrations of P–PO_4 that occur in the lake during an annual cycle [16]. The cultures were shaken by hand everyday to assure homogenization inside the flask and to avoid sedimentation or flotation

of the cyanobacteria. For each culture condition, the cell density was estimated in 3 randomly selected Erlenmeyer flasks out of the 15 flasks by determining the optical density (OD) at 570 nm and counting the filaments as described above. The OD was measured using a Perkin Elmer® Lambda 2S spectrophotometer.

Microcystin Extraction. Samples from the lake and from the cultures were filtered on a 1-μm filter (Nucleopore, Whatman). Microcystins were extracted from 2 L of lake water and from 25 to 100 mL of cultures, depending on the cell density. The cultures were taken from 3 randomly selected flasks among the 15 Erlenmeyer flasks for each condition. The intracellular microcystins were extracted from the cells in MeOH 75% after sonicating for 20 min. This step was performed twice. The samples were then centrifuged at 20,000 rpm for 10 min to remove all particles from the supernatant. The supernatant was then filtered through a 0.22-μm syringe filter (Nalgene, VWR) and evaporated using a Speedvack (Speed vac® Plus SC110A, Savant) device. The extracts obtained were dissolved in MeOH 50% prior to HPLC analysis. For the extracellular detection of MCs, filtrates of the cultures (100 mL) were concentrated on a Bakerbond SPE C18 cartridge according to [35]. Extracts were stored at −20°C prior to analysis.

High-Pressure Liquid Chromatography Analysis. The high-pressure liquid chromatography (HPLC) system consisted of a 600 pump (Waters) connected to a six-port Rheodyne valve equipped with a 20-μL injection loop. UV detection was performed using a PDA 496 photodiode-array detector (Waters). Separations were carried out using a 250 × 3 mm i.d. column packed with 5-μm reversed phase SYMETRY C_{18} silica (Waters). The mobile phase, which was run at a flow rate of 1 mL/min, consisted of the following mixtures of MilliQ–water–0.5% v/v trifluoroacetic acid (A) and acetonitrile (B): 30% of B at 0 min to 50% of B at 40 min [5]. We used the elution conditions of [30] to identify the MC variants. MC peaks were identified on the basis of their characteristic absorption spectra (maximum absorption at 238 nm) and by their retention times.

HPLC–Electrospray Ionization–Mass Spectrometry Analysis. The instrumentation used for micro-liquid chromatography (LC) consisted of a 1100 Series pump (Agilent Technologies, Waldbronn, Germany) connected to an accurate 1/10 microflow splitter (LC Packings, Amsterdam, The Netherlands). For direct injection, the outlet was linked to a four-port Valco CIAW valve (VICI, Valco Europe, Switzerland) with a 1-μL injection loop. An LC-5A preconcentration pump (Shimadzu, Kyoto, Japan) was used for on-line preconcentration, and the precolumn consisted of a microprecolumn kit (LC Packings). UV detection was performed either with an SPD-10A detector (Shimadzu) fitted with a U-shaped microcell (LC Packings) or with an SPD-M10A photodiode array detector (Shimadzu) fitted with a microcell with an internal volume of 140 nL (prototype LC Packings). On-line micro-solid phase extraction (SPE)/micro-LC/electrospray ionization (ESI)/MS/MS analyses were carried out on a VG Quattro (Fisons Instruments, VG Biotech, Altrincham, United Kingdom) triple quadrupole fitted with an electrospray ion source. The ESI/MS and ESI/MS/MS signals were optimized using the four MC standards (-LR, -RR, -LA, and -YR) in a mixture of 50% acetonitrile–50% H_2O containing 0.03% trifluoroacetic acid (TFA) (v/v). A potential difference of 2.5 kV was applied, and the cone voltage was adjusted to 35 V for -RR and -dMeRR, to 40 V for -LA, and to 70 V for -LR, dMe-LR, -YR, and for MC 1030.7 Da. Nitrogen heated to 100°C was used as the drying gas. Data analysis was carried out using MassLynx 3.3V. Full scan spectra were acquired in the positive ion peak centroid mode over the mass range of m/z 100–1200 for 4.5 s. Multiple reaction monitoring mode was obtained with the fragmentation of $[M + H]^+ \rightarrow 135$ m/z for all MC or $[M + 2H]^{2+} \rightarrow 135$ m/z for MC-RR and -dMeRR. The energy of collision applied was 200 eV. The μSPE cartridge was a PLRP-S 15 × 1 mm i.d. 5 μm (LC Packings), through which 5 mL of the sample was percolated at 0.5 mL/min. Analytical micro-LC separations were performed using a 250 × 1 mm i.d. 5-μm reversed-phase Hypersil C18 BDS (LC Packings) or a 100 × 1 mm i.d. reversed-phase Equisil C18 BDS (C.I.L. Cluzeau, Sainte Foy la Grande, France). The elution gradient employed mobile phase A, consisting of acetonitrile, and mobile phase B, consisting of water containing 0.03% TFA. The gradient was 30–50% A from time 0 to 30 min and 70% A at 40 min. A second gradient was used to separate the minor variant: 20–40% A from time 0 to 30 min. The flow rate was 50 μL/min after the microflow splitter and entered without any split in the ESI/MS/MS. The UV detection was set to 238 nm.

Microcystin Standards. MC-LR, -RR, -YR, and -LA standards were purchased from Sigma Chemical (St. Quentin Fallavier, France) and Calbiochem (VWR, Fontenay-sous-Bois, France). Qualitative standards of [D-Asp^3] MC-RR, [Dha^7] MC-RR, and [D-Asp^3, Dha^7] MC-RR were kindly provided by Dr. J. Rapala (Finnish Environment Institute, Helsinki). A field sample from Lake Weida containing [D-Asp^3, Dhb^7] MC-RR [9] was kindly provided by Dr. J. Fastner (Federal Environmental Agency, Berlin).

Characterization of Growth and MC Production in the Experiments. The growth rate μ (day^{-1}) during the

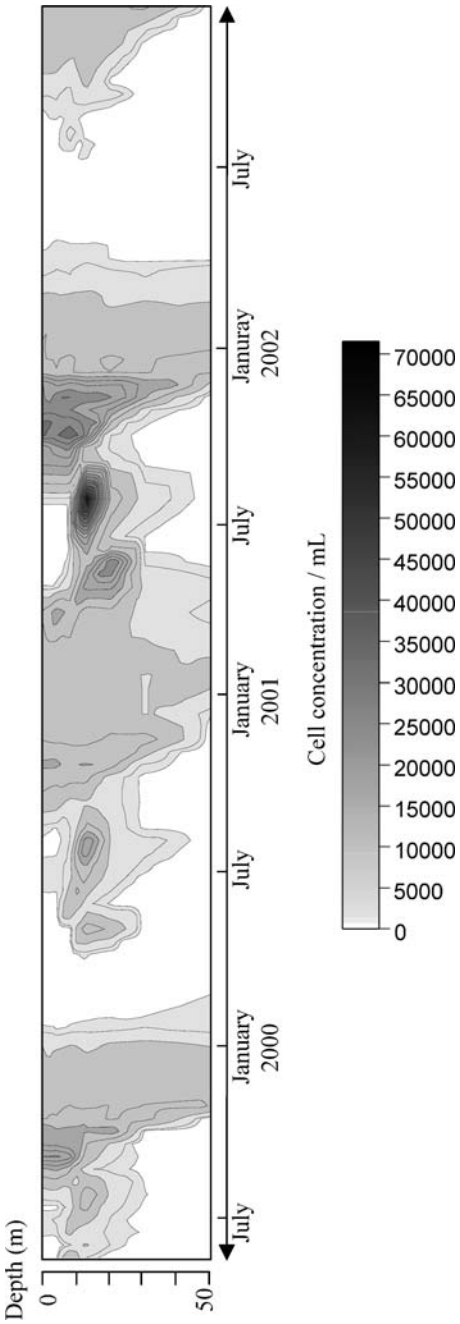

Figure 1. Abundance (cell/mL) of the *P. rubescens* population in Lac du Bourget from January 1999 to January 2003 at depths between 0 and 50 m.

exponential phase was calculated using the following equation:

$$B = B_0 e^{\mu t}$$

where B is the biomass at time t and B_0 is the biomass of the inocula, expressed as the OD.

The MC production rate (day^{-1}) was calculated using the following equation:

$$MC = MC_0 e^{\mu t}$$

where MC is the intracellular MC concentration at time t and MC_0 is the intracellular MC concentration of the inocula.

Results

Distribution and Dynamics of P. rubescens in Lac du Bourget. The vertical distribution and dynamics of *P. rubescens* in Lac du Bourget were characterized by three distinct phases (Fig. 1). The first phase was recorded just after the thermal stratification of the lake, from late spring to the end of September. During this period, *P. rubescens* was located in the metalimnic layer, between 12- and 18-m depth, and the highest densities recorded during the years 1999–2002 ranged from 6000 to 77,400 cells mL^{-1} (Table 1). During the second phase (from October generally to November), *P rubescens* moved upwards in the epilimnion (Fig. 1). Depending on the biomass level reached by the cyanobacterium in the stratified layer in summer, its colonization of the epilimnion could cause impressive red discoloration of the surface water, as it did in September 2001, when the cell density reached 47,600 cells mL^{-1}. The highest densities reached in the epilimnion in the years 1999–2002 ranged from 6270 to 47,600 cells mL^{-1} (Table 1). During the final phase (from December to March), *P. rubescens* gradually colonized the entire water column (from 0- to 50-m depth and beyond, not shown), and the water column was mixed to deeper and deeper levels in response to falling temperatures and the effect of wind. The cell concentration generally fell to very low values, except in 2001 when high values were also found in spring (Fig. 1).

Identification of MC Variants. Intracellular extracts from field samples of *P. rubescens* analyzed by HPLC-DAD revealed four main peaks with the following retention times: 9.2, 9.7, 16.2, and 21.5 min and with UV spectra typical of MC. We failed to separate the first two MC peaks completely, despite trying various gradients. The fourth microcystin had the same retention time as the standard for the MC-LR. LC/MS and LC/MS/MS analyses allowed us to confirm the presence of four MCs with the characteristic ion peak at $[M + H\text{-}134]^+$ in sparse mode and the ion m/z 135 corresponding to fragmentation of (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,

Table 1. Highest cell densities recorded in the metalimnic and the epilimnic layers in Lac du Bourget from 1999 to 2002

	Cell density (cell mL^{-1})			
Studied years	*1999*	*2000*	*2001*	*2002*
Metalimnion	15,120	13,200	77,400	6000
Epilimnion	35,280	16,560	47,600	6270

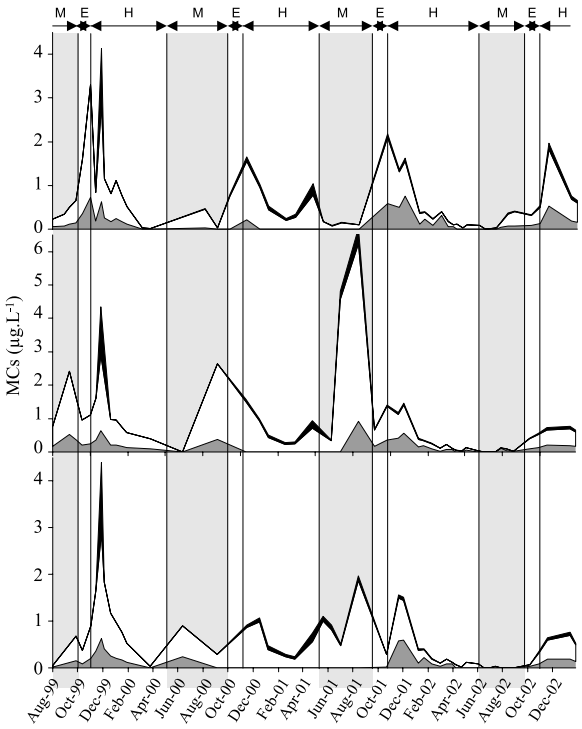

Figure 2. MC concentrations in natural *P. rubescens* cells in Lac du Bourget at depths of 10 (A), 15 (B), and 20 m (C) from 1999 to 2003. Concentrations of the three main variants, [D-Asp^3] MC-RR (■), [D-Asp^3, Dhb^7] MC-RR (□), and [D-Asp^3] MC-LR (■), were added to give the total MC concentration. All the concentrations were expressed in µg L^{-1} LR eq. (M = metalimnic phase, E = epilimnic phase, H = homogeneous phase).

6-dienoic acid (ADDA). We then identified the first two MCs as demethyl MC-RRs, both with a molecular mass of 1023 Da ($[M + H]^+$ 1024) indicating a single demethylation (Mw MC-RR = 1037 Da), unlike [D-Asp^3, Dha^7] MC-RR, which is a "double-demethyl" MC (Mw = 1009 Da). Using the conditions described by Lawton *et al.* [24], the three demethyl MC-RRs can be separated (Fastner, personal communication). [D-Asp^3] MC-RR appeared to be the first MC to be eluted, closely followed by [D-Asp^3, Dhb^7] MC-RR. The [Dha^7] MC-RR peak also followed close behind the previous MC and was coeluted with MC-RR. When the samples had been spiked with purified standards of [D-Asp^3] MC-RR and [Dha^7] MC-RR on one hand, and with a sample field containing [D-Asp^3, Dhb^7] MC-RR on the other hand, we were able unambiguously to identify the first peak as [D-Asp^3] MC-RR and the second peak as [D-Asp^3, Dhb^7] MC-RR. The two other MCs were identified as MC-YR or -HtyrR (Mw = 1044 Da) and dMe-LR (Mw = 980 Da), but no further investigation was performed because they constituted only small percentages of the MCs.

Cultured extracts of the strain TCC 29 revealed a simpler microcystin profile (intra- and extracellular) with only one MC identified as [D-Asp^3] MC-RR on the basis of its mass spectrum and HPLC retention times.

Annual Fluctuations of P. rubescens MC Concentrations in Lac du Bourget. As the quantitative standards for the different MCs identified were not available, we were not able to determine the absolute concentrations of each MC. The peak areas were therefore expressed individually in terms of the MC-LR standard and then added together to give a total value in terms of the MC-LR equivalent (MC-LR eq). When MC-YR was detected, it was always present as traces and was not therefore quantified.

Total MC concentrations were very often greater than 1 µg L^{-1} in 1999, 2000, and 2001 (Fig. 2). The highest concentrations were recorded in November 1999 and August 2001, with values of 4.3 and 6.7 µg L^{-1}, respectively. Values in 2000 and 2002 were lower, with maximum concentrations of 1.97 and 2.64 µg L^{-1}, respectively. The general microcystin pattern in the Lac du Bourget displayed the same annual pattern at all the depths sampled, with a progressive rise in the MC concentration during the summer, and maximum values recorded between August and November, generally at a depth of 15 m. At the 20-m depth, microcystin concentrations were always lower than at 10 or 15 m, except in November 1999.

Throughout the period of interest, the two demethyl MC-RR always corresponded to around 93% of the MC produced by *P. rubescens* (Table 2) for all the depths and

Table 2. Proportions of the various MC variants found in extracts of Lac du Bourget at different depths and during different periods of the year corresponding either to stratified conditions (S) or homogeneous conditions (H) in the water column (SD = standard deviation)

	All depths			*Depth = 10 m*			*Depth = 15 m*			*Depth = 20 m*		
		S	*H*		*S*	*H*		*S*	*H*		*S*	*H*
Proportion (% ± SD)	*n = 192*	*n = 60*	*n = 132*	*n = 51*	*n = 17*	*n = 34*	*n = 49*	*n = 16*	*n = 33*	*n = 48*	*n = 14*	*n = 34*
Total dMeRR	93 ± 11	95 ± 6	90 ± 9	93 ± 14	96 ± 7	91 ± 16	95 ± 6	97 ± 3	92 ± 6	95 ± 7	95 ± 4	93 ± 7
[D-Asp^3] MC-RR	16 ± 22	10 ± 24	12 ± 15	17 ± 21	7 ± 11	22 ± 23	25 ± 27	14 ± 14	23 ± 20	17 ± 21	6 ± 16	21 ± 22
[D-Asp^3, Dhb^7] MC-RR	75 ± 22	87 ± 24	83 ± 15	82 ± 21	92 ± 11	73 ± 28	75 ± 25	68 ± 38	77 ± 20	83 ± 21	94 ± 16	79 ± 22

periods considered. The main variant was [D-Asp^3, Dhb^7] MC-RR, which ranged from 55 to 100% (mean value 75%, data not shown) of the two demethyl MC-RRs. Once again, the depth and period of sampling considered had no impact on the ratio between the two demethyl MC-RRs (Mann–Whitney U test, $p < 0.01$).

Relationships between MC Concentrations and P. rubescens Cell Densities. The correlation between the total cell concentration of MC and *P. rubescens* densities was statistically significant when all the data were taken into consideration ($r^2 = 0.60$, $n = 192$, $p < 0.01$). The correlation was ever closer if we only considered the period when the cyanobacteria were stratified in the metalimnic layer, i.e., from May to September ($r^2 = 0.72$, $n = 60$, $p < 0.01$ for total MC concentration; $r^2 = 0.84$, $n = 34$, $p < 0.01$ for [D-Asp^3] MC-RR; $r^2 = 0.67$, $n = 60$, $p < 0.01$ for [D-Asp^3, Dhb^7] MC-RR; and $r^2 = 0.84$, n=39, $p < 0.01$ for [D-Asp^3] MC-LR; Fig. 3).

Cellular quotas of MC (expressed in pg MC-LR equivalent per cell) ranged from 0.1 to 0.3 pg cell^{-1} (Table 3). This quota appeared to be slightly higher during the stratified period (0.23 ± 0.42 pg cell^{-1}) than during the nonstratified period (0.12 ± 0.16 pg cell^{-1}), but these values were not statistically different (Mann–Whitney U test, $p > 0.05$).

Table 3. MC cellular quotas at different depths and different times of year corresponding either to stratified conditions (S) or homogeneous conditions (H) in the water column (SD = standard deviation)

	MC cellular quota (pg cell^{-1} ± SD)		
	S + H	*S*	*H*
All depths (n = 192)	0.16 ± 0.27	0.23 ± 0.42	0.12 ± 0.16
10 m (n = 51)	0.15 ± 0.17	0.18 ± 0.20	0.13 ± 0.16
15 m (n = 49)	0.16 ± 0.23	0.14 ± 0.08	0.17 ± 0.26
20 m (n = 48)	0.13 ± 0.16	0.10 ± 0.08	0.09 ± 0.06

Effects of P–PO_4 Concentrations on the Growth Rate and MC Production of P. rubescens. Growth curves were expressed in cell mL^{-1} (Fig. 4) after conversion of the OD measurements at 570 nm, based on the fact that these two parameters displayed a significant positive correlation ($r^2 = 0.88$, $n = 28$, $p < 0.01$). *P. rubescens* TCC 29 grew at all the phosphate concentrations tested.

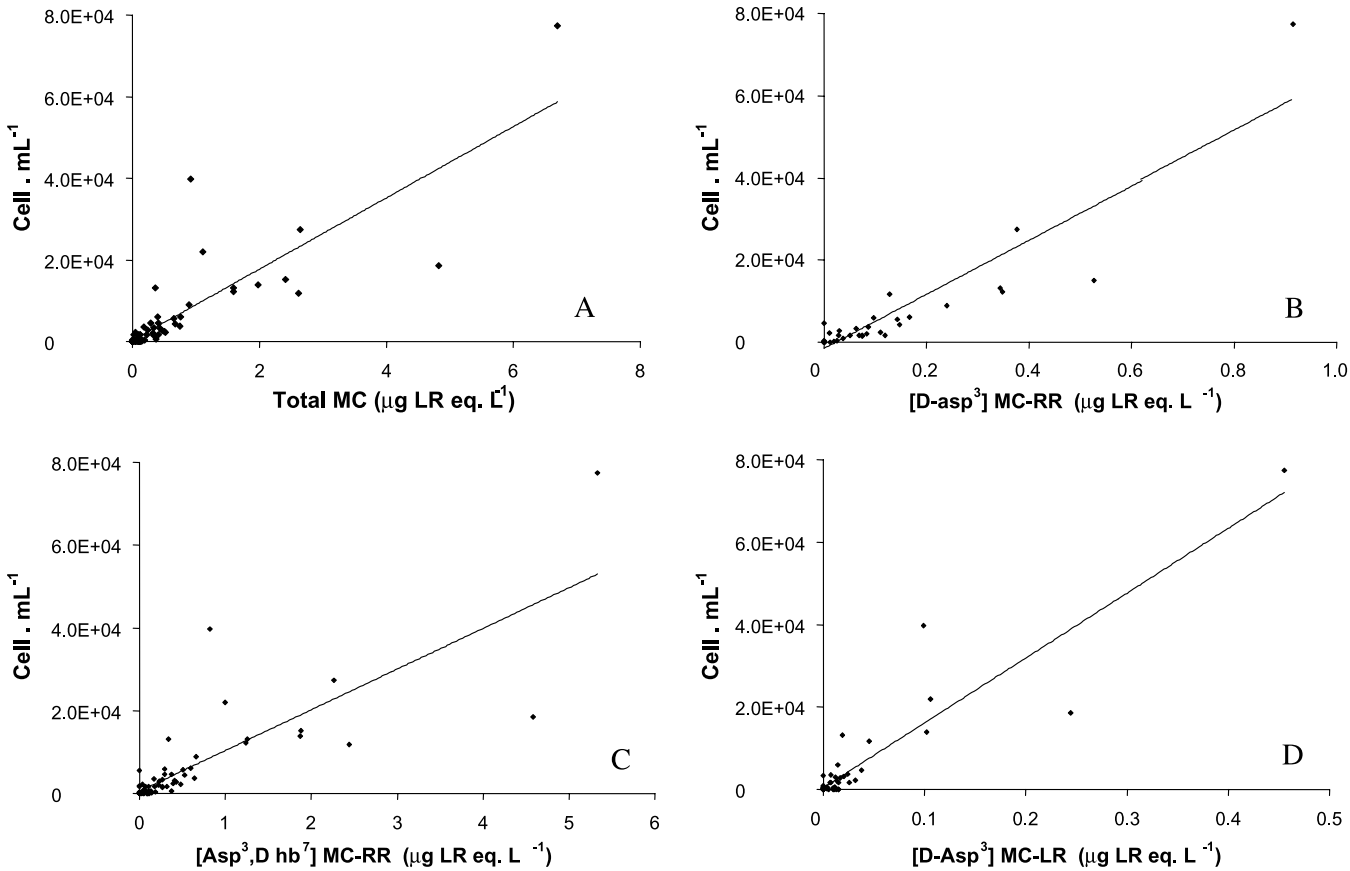

Figure 3. Correlations between the density of *P. rubescens* and MC concentrations during the stratified periods in the water column. (A) Total MC ($r^2 = 0.72$, $n = 60$, $p < 0.01$); (B) [D-Asp^3] MC-RR ($r^2 = 0.84$, $n = 34$, $p < 0.01$); (C) [D-Asp^3, Dhb^7] MC-RR ($r^2 = 0.67$, $n = 60$, $p < 0.01$); (D) [D-Asp^3] MC-LR ($r^2 = 0.84$, $n = 39$, $p < 0.01$).

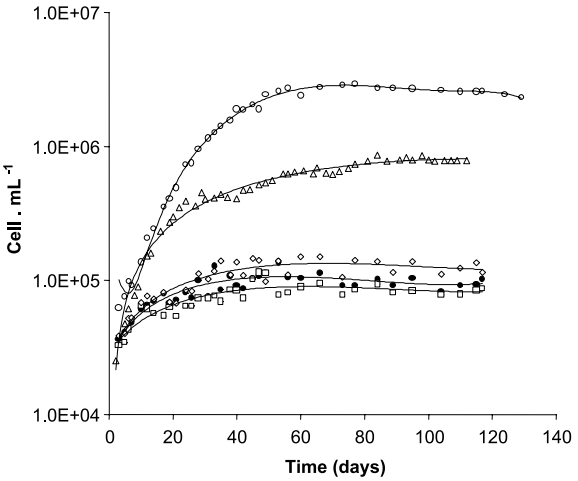

Figure 4. Growth curves of *P. rubescens* strain TCC 29 for various initial phosphate concentrations (Z0 ●, Z1 □, Z2 ◊, Z3 △, and Z ○).

Cultures in Z medium exhibited the same growth rate as in Z3 during the exponential phase ($\mu = 0.14$ day^{-1}), but this phase lasted for 21 days in Z, i.e., for twice as long. Until day 56, when the stationary phase began, the mean growth rate remained 0.04 day^{-1}. The density of *P. rubescens* reached its highest value of 3×10^6 cells mL^{-1} at this phosphate concentration. No marked decrease in biomass was observed until after day 117. Cultures in Z3 showed an initial growth rate of around 0.14 day^{-1}, and then growth declined from day 12 ($\mu = 0.05$ day^{-1}). The stationary phase was only reached from about day 80, with a maximum biomass of around 8×10^5 cells mL^{-1}. Cultures in Z0, Z1, and Z2 all displayed similar growth-rates, with an exponential phase lasting until day 12 ($\mu =$ 0.08 day^{-1}), followed by a decrease ($\mu = 0.02$ day^{-1}) until the stationary phase was reached around day 40. The maximum densities approximated 1–1.5 $\times 10^5$ cells mL^{-1}.

P. rubescens strain TCC 29 only produced one MC variant, [D-Asp^3] MC-RR. To permit comparisons with the field data, microcystin concentrations were expressed in MC-LR equivalents. Whatever the initial phosphate concentrations tested, all cultures displayed the same pattern with intracellular MC correlating closely with cell densities ($r^2 = 0.85$, $n = 25$, $p < 0.01$), and extracellular MC only detectable during the senescence phase, with the exception of the Z3 culture (Figs. 5A, C). The intracellular MC content reached its highest values under the Z culture conditions, peaking at 1133 μg L^{-1} at day 77. The decline of the intracellular concentrations coincided with the rapid rise of extracellular MC from 28 to 1600 μg L^{-1} between days 117 and 129 (Fig. 5A). Under Z3 culture conditions, the intracellular MC content also increased until it reached its maximum at day 31, with a higher value (136 μg L^{-1}) than for the Z0 to Z2 cultures (Fig. 5B). No extracellular MC was ever detected in the Z3 cultures (Fig. 5C). Under the Z0, Z1, and Z2 culture conditions, the intracellular MC content increased slightly up to day 31 (reaching a peak of 30 μg L^{-1}) and then declined (Fig. 5B). The extracellular MC only rose above the threshold of detection on day 117 (approx. 20 μg L^{-1}; Fig. 5C).

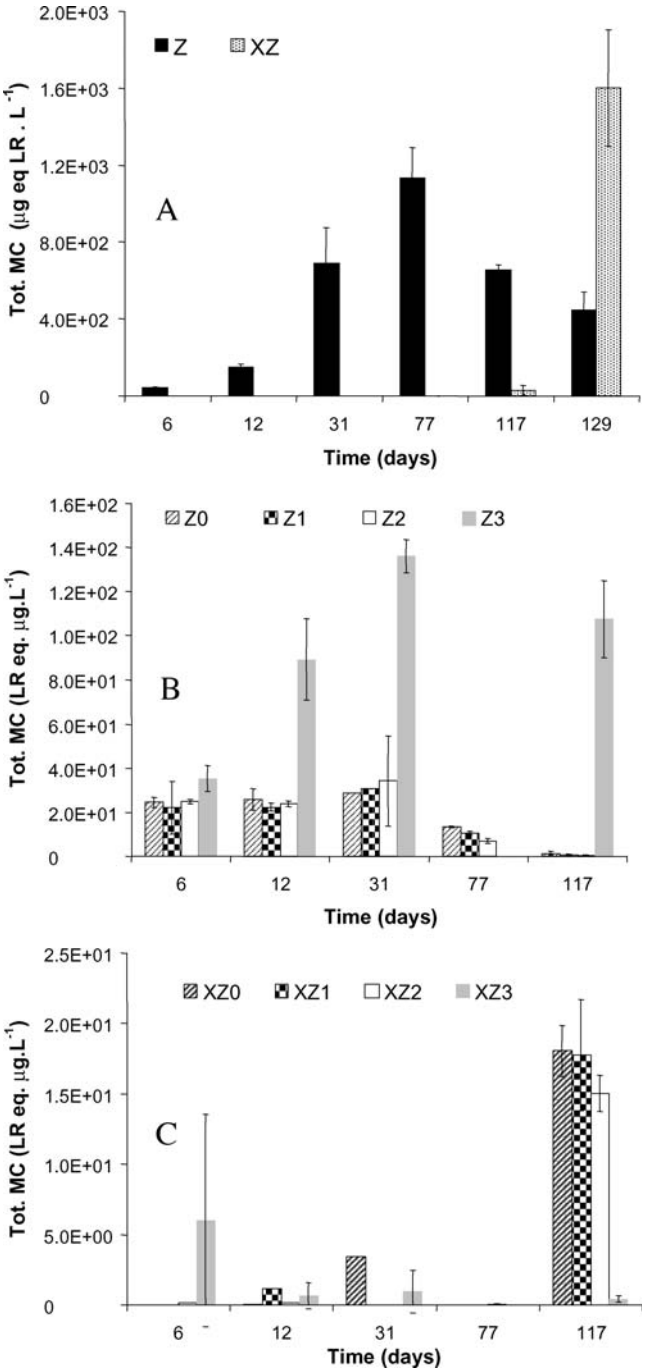

Figure 5. Evolution of the intracellular and extracellular MC concentrations (expressed as MC-LR equivalents) for the five different P–PO_4 concentrations tested in cultures of the *P. rubescens* strain TCC 29-1. Changes in the intracellular (Z) and extracellular (XZ) MC concentrations in Z medium (A). Changes in the intracellular (B) and extracellular (C) MC concentrations in the Z0, Z1, Z2, and Z3 media.

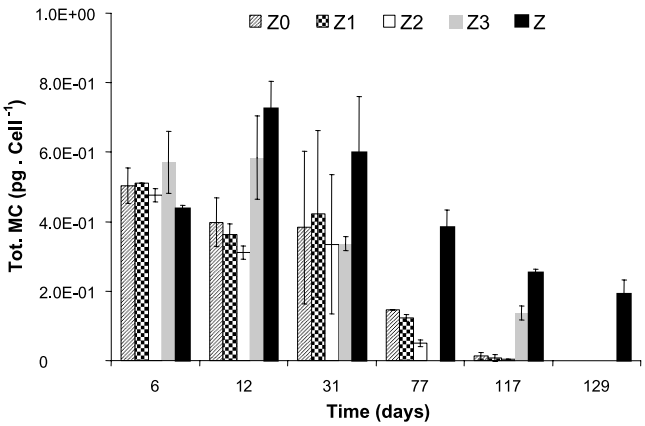

Figure 6. Changes in the MC cellular quotas in the *P. rubescens* TCC 29-1 strain for the five different P–PO_4 concentrations tested in culture.

The highest intracellular MC quota (expressed in MC-LR eq.) reached was 0.7 pg $cell^{-1}$ (Fig. 6). These quotas ranged from 0.3 (±0.02) to 0.7 (±0.08 pg) $cell^{-1}$ during the exponential growth phase for all culture conditions. A significant correlation ($r^2 = 0.40$, $n = 25$, $p < 0.05$) was found between the MC quota and culture growth rates.

The MC production rates (in day^{-1}) were also calculated for the five different culture conditions. A highly significant correlation was found between MC production rates and growth rates ($r^2 = 0.75$, slope = 1.19, $n = 25$, $p < 0.01$; Fig. 7).

Discussion

The main objectives of this study were to evaluate the qualitative and the quantitative changes in *P. rubescens* microcystin production at different depths in Lac du Bourget and the relationships between these changes and the population dynamics of this species. More specifically, the effect of changes in a major parameter (the concentration of phosphorus) on population growth and microcystin production was tested in the laboratory.

First, our study revealed that in Lac du Bourget, *P. rubescens* produces two main demethyl-RR microcystins, [D-Asp^3] MC-RR and [D-Asp^3, Dhb^7] MC-RR, and probably one demethyl-LR microcystin. According to the literature concerning the MC variants produced by *P. rubescens*, a [D-Asp^3] MC-LR may have been involved [9]. The absolute identification of these variants would require further 2D NMR analyses of purified samples [2, 9, 37], but the liquid chromatography analysis coupled to mass spectrometry plus the data available in the literature for *P. rubescens* strongly supported our identification. Indeed, [D-Asp^3, Dhb^7] MC-RR is always reported as being the main variant produced by this species [2, 9]. The [D-Asp^3] MC-RR variant was not identified in Lake Zürich [2], but it has been detected as a minor constituent in German lakes [9], where it never exceeds 19% of the total MC. In addition, this variant was also reported to be the main MC in two "red-colored" *Oscillatoria agardhii* isolated from Finnish lakes [26]. Regarding the other minor variants, the presence of demethylated forms of MC-YR and MC-LR has also been detected in the German and the Swiss populations, as reported in the studies referred to above.

Second, it appears that the ratio between the various MC variants was globally the same all year round and at the different sampling depths, as previously reported for *P. rubescens* [9], but also for *M. aeruginosa* [32]. When variable ratios between MC variants have been reported in the literature, this has been associated with variations in the evolution of the relative proportions of MC-producing strains, but this has never been reported for *P. rubescens* [5, 17, 20, 46]. The fact that the strain isolated from Lac du Bourget that we used in our experiments only produced [D-Asp^3] MC-RR shows that different strains producing the other MC variants identified are probably also part of the natural population in this lake. However, the truth of such a statement could only be demonstrated after direct analyses of several single filaments by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry [21, 46].

It has recently been reported by Kurmayer *et al.* [21] that in two Austrian lakes, the proportions of *P. rubescens* filaments with and without MC remain stable from May to September. In addition, it was found that all the *P. rubescens* filaments have the MC synthetase genes, and that the co-occurrence of MC producers and non-MC producers was only due to the inactivation of the *mcy* genes. On the other hand, for *Microcystis* spp.

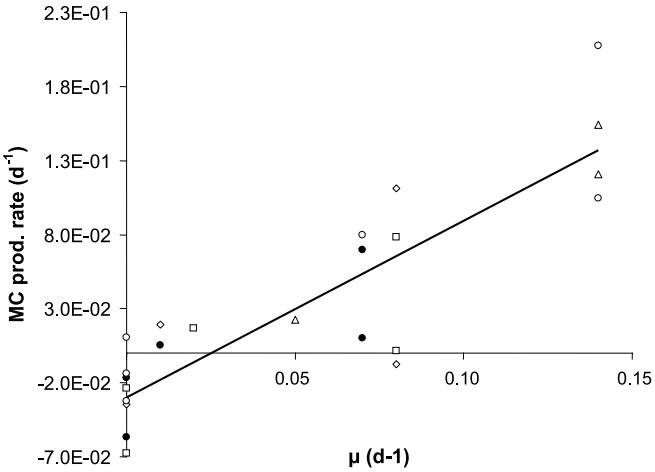

Figure 7. Correlations between the total MC production rate and the corresponding growth rate of *P. rubescens* strain TCC 29-1 at the five different P–PO_4 concentrations tested in culture ($r^2 = 0.75$, $n = 25$, $p < 0.01$; Z0 ●, Z1 □, Z2 ◊, Z3 △, and Z ○).

[20, 46] or *P. agardhii* [21], the co-occurrence of MC producers with non-MC producers seems to be due both to the presence/absence of *mcy* genes and to their inactivation.

The variations in the total microcystin cell content ranged from 0.1 to 0.3 pg $cell^{-1}$ under natural conditions and from 0.3 to 0.7 pg $cell^{-1}$ in our laboratory experiments. When these data are compared with those in the literature, we found that 1.5- to 3-fold changes in the microcystin cell content had also been reported by Long *et al.* [25] and Wiedner *et al.* [48], suggesting that all microcystin-producing strains may exhibit cellular microcystin contents within a fairly narrow range.

Third, both the field and experimental studies have shown a significant correlation between *P. rubescens* biomass and MC concentrations. More particularly, the field survey showed that this correlation was closer during the first phase of the *P. rubescens* annual cycle, when the cyanobacterium was located in the metalimnic layer. At this time, the *P. rubescens* cell concentration was always increasing, which means that the cyanobacteria were growing. It is interesting to note that cultures containing phosphate concentrations similar to those recorded in the lake (i.e., 0.03 mg L^{-1}) exhibited a growth rate (0.08 day^{-1}; Fig. 4), which was lower than the field data, which were always up to 0.1 day^{-1} during this stratified period (data not shown). This seems to reinforce the hypothesis that local nutrient pulses in the metalimnion of Lac du Bourget allow the growth of *P. rubescens* to occur [16]. When the cyanobacterium spread throughout the entire epilimnic layer and then throughout the whole water column, this correlation was not as close, but always remained statistically significant. At this time, there was generally a decrease in the cell concentration of *P. rubescens*, probably due to the dilution of the cells in a larger volume of water, as well as to cell death and sedimentation. These findings are entirely consistent with those obtained in our experimental study, showing that whatever the culture conditions, the MC intracellular quota was always of the same order of magnitude during the exponential growth phase. On the other hand, during the stationary phase, and more significantly during senescence, the intracellular content fell as a result of the release of extracellular MC. This finding was not observed in the Z3 medium in which extracellular MC did not increase at day 117, whereas the intracellular MC displays a classic pattern with decreasing concentrations at the end of the steady state (Fig. 4). MC degradation by bacteria has now been demonstrated [27] and could be the reason for the disappearance of MC from the extracellular medium. A parallel study of the dynamics of eubacterial populations associated with these *P. rubescens* cultures (Dorigo, personal communication) showed a marked increase in the abundance of these eubacteria during the steady state and senescence phases and so provided further evidence in favor of this hypothesis.

All the findings of this study suggest that MC production by *P. rubescens* in Lac du Bourget is not directly influenced by environmental parameters, such as nutrient concentrations, temperature, or light conditions. Indeed, despite the changing environmental conditions experienced by the *P. rubescens* population from April to December, there were no significant changes in the MC content of the cells. On the other hand, there was a significant correlation between the rate of MC production and the growth rate of *P. rubescens* (Fig. 7). This strongly suggests that environmental parameters may have an indirect effect on MC production, as a result of their direct impact on cellular growth rates. This finding is consistent with several papers dealing with *M. aeruginosa* [25, 31, 43, 48], which have suggested that microcystin production was directly linked to the cell division rate rather than being induced by any direct effect on the metabolic pathways of microcystin production in response to environmental factors such as nutrients. Concerning the influence of light, Wiedner *et al.* [48] concluded that photosynthetically active radiation has a positive effect on microcystin production and content up to the point where the maximum growth rate is reached. These authors assumed that the contradictory findings reported in the literature can be largely explained by differences in the experimental protocols and by the fact that the cellular microcystin contents are expressed in terms of different cell parameters.

In conclusion, data are very scarce for the *Planktothrix* genus, and this is the first laboratory study reporting the potential effect of environmental parameters on MC production by *P. rubescens*. In addition, the laboratory experiments gave us some information about the role of phosphorus in growth and MC production, which is of particular interest in the case of *P. rubescens*, because this species is usually found in mesotrophic lakes where phosphorus concentrations can be a limiting factor during a large part of the year. Finally, according to Orr and Jones [31], the direct linear correlation found between cell division and microcystin production rates, regardless of environmental factors, suggests that microcystin is not a secondary metabolite.

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