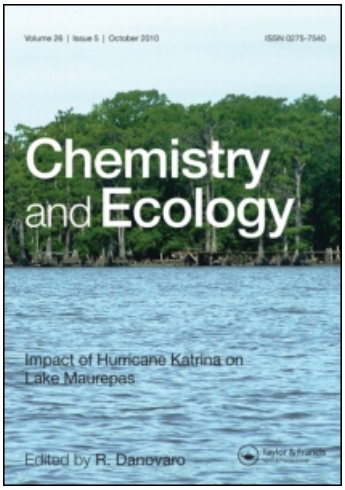

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Growth responses of *Microcystis ichthyoblabe* Kützing and *Anabaena aphanizomenoides* Forti (cyanobacteria) under different nitrogen and phosphorus conditions

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Experiments in batch cultures under controlled sub-optimal light and temperature conditions were undertaken to determine the effect of nitrogen, phosphorus and N:P ratios on the growth of *Microcystis ichthyoblabe* Kütz. 1843 and *Anabaena aphanizomenoides* Forti 1912, two toxic cyanobacteria forming blooms from Oued Mellah lake. Phosphorus experiments show that densities of *M. ichthyoblabe* and *A. aphanizomenoides* increased differently in the various media. Under non-limiting phosphorus conditions (1000–6960 $\mu g\,P\,l^{-1}$), 5–7 days of exponential growth was observed, while in P-free and in P-deficient media (0–500 $\mu g\,l^{-1}$), the growth was limited. As with phosphorus experiments, cell growth of *M. ichthyoblabe* was substantially favoured under high nitrate concentrations (50–84 $mg\,l^{-1}$), whereas cultures under N-free or N-deficient conditions (0–10 $mg\,l^{-1}$) seemed to be limited. Nitrate-nitrogen at all tested concentrations was not limiting for the growth of *A. aphanizomenoides* cultures, which reached high density during an exponential growth of 8–9 days. Under low nitrate concentrations (0–5 $mg\,l^{-1}$), an increased number of heterocysts was observed. There was a markedly diminished growth with the lower N:P ratio experiments ($\leq$5) only for *Microcystis* and on the highest N:P ratio experiments ($\geq$30) for both *Microcystis* and *Anabaena aphanizomenoides*.

Keywords: cyanobacteria; *Microcystis ichthyoblabe*; *Anabaena aphanizomenoides*; nitrogen, phosphorus; N:P ratio; ecophysiology

1. Introduction

In Morocco, like in many other areas of the world, toxic cyanobacterial blooms are common events [1,2]. Oued Mellah reservoir, a semiarid man-made lake, is a problematic reservoir in terms of toxic cyanobacteria [3–5]. During a hydrobiological study in 1997–1999, intensive hepatotoxic blooms of *Microcystis ichthyoblabe* Kütz. 1843 and *Anabaena aphanizomenoides* Forti 1912 were recorded in late spring and summer. Several hypotheses have been proposed to explain the success of cyanobacteria in this water-body, including high water temperature, low water

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transparency, grazing resistance, buoyancy regulation, efficient nutrient assimilation and nutrient storage. Nutrient availability plays an important role in controlling structure and biomass of phytoplanktonic communities and seasonal species succession. Nevertheless, the relationship between available P and N and cyanobacteria dynamics is species specific. Spróber et al. [6] showed that at excess phosphorus supply (5 mg PO4-P l^{-1}) the biomass of *Cylindrospermopsis raciborskii* Woloszynska 1912, occurred. In another subtropical reservoir, Posselt and Burford [8] showed that *C. raciborskii* growth was P limited and did not react much to ammonium injection. *Microcystis* species also react in a similar way to P increase in temperate lakes [9].

Field observations in the Oued Mellah lake indicated that blooms dominated by *M. ichthyoblabe* and *A. aphanizomenoides* frequently occur in relatively high N:P ratios, which is not consistent with literature data. Recently we showed that light and temperature have an important influence on the dynamics of *Microcystis ichthyoblabe* Kütz. 1843 and *Anabaena aphanizomenoides* in laboratory controlled experiments [10]. The aim of the present study was to investigate the different behaviour of the isolated strains of *M. ichthyoblabe* and *A. aphanizomenoides* in N and P deficient and sufficient media, and in different N:P ratios under controlled laboratory conditions.

2. Materials and methods

The cyanobacterial strains of the species *Microcystis ichthyoblabe* and *Anabaena aphanizomenoides* were isolated from the hypertrophic lake of Oued Mellah (33°30′N – 07°20′W), Morocco, during a summer bloom in 1999. A stock culture of each species was maintained in liquid Z8 medium [11] by regular subculturing (every 3–5 days). The cyanobacteria were grown in a culture room at 25 ± 2 °C under continuous irradiation of 35 μE m^{-2} s^{-1} and suspended by gentle aeration with filtered air.

Experiments in batch cultures (250 ml) were conducted to determine the effect of nitrogen, phosphorus and N:P ratios on the growth of these species. All cultures were kept in a culture room at a temperature of 28 ($\pm$2) °C with a 15 light : 9 dark cycle under an irradiance of 35 μE m^{-2} s^{-1} provided by 40 W cool-white fluorescent lamps, and continuously aerated with filtered air. This temperature was chosen because it is the highest recorded in the Oued Mellah reservoir, where the strains were isolated. Experiments with *M. ichthyoblabe* and *A. aphanizomenoides* lasted 10 days and were conducted in Z8 medium with modification of N or P concentrations or an N:P ratio. Prior to each experiment, the stock cultures were concentrated using Millipore filters (0.45 μm), washed with P- and/or N-free Z8 medium according to the N, P or N:P test. Each 250 ml aliquot of the prepared medium was inoculated with *M. ichthyoblabe* or *A. aphanizomenoides* stock cultures, resulting in a starting density of 3.1×10^5 *Microcystis* cell ml^{-1} or 5.7×10^3 *Anabaena* trichomes ml^{-1}. These experiments were conducted with the initial concentrations of phosphorus, nitrogen and N:P ratios as shown in Table 1. K_2HPO_4 was used as the only phosphorus source in the cultures, while $NaNO_3$ and $Ca(NO_3)_2$, $4H_2O$ were supplied at a molecular ratio of 20:1 as a source of nitrogen. In order to maintain the ionic strength of the medium, KCl was added in amounts equivalent to the phosphate substracted from the medium, and NaCl when nitrate was not present. In experiments 1 to 8, N was provided at an optimal concentration of 84 mg $N.l^{-1}$ (Z8) and P varied from 0–6969 μg$P\,l^{-1}$ (Table 1). In experiments 9 to 16, P was provided at a optimal concentration of 6969 μg$P\,l^{-1}$ (Z8) and >N varied from 0–84 mg $N.l^{-1}$ (Table 1).

Sub-samples for cyanobacteria quantification were taken daily from each vial and preserved with Lugol's iodine solution. Quantification was done with a hemocytometer after sonication (50 kHz, 10s) for *M. ichthyoblabe* and with an inverted microscope for *A. aphanizomenoides*. The integrity of the cyanobacteria cells after sonication was confirmed in the laboratory prior the experiments. All experiments were performed using two replicates for each growth condition and the results are presented as average values.

Table 1. Initial nitrogen and phosphorus concentrations and N:P ratios used in the different experiments.

Experiment	Phosphorus (μg P l^{-1}) Nitrogen (84 mg N.l^{-1})	Experiment	Nitrogen (mg N l^{-1}) Phosphorus (6960 μg P l^{-1})
1	0	9	0
2	5	10	0.05
3	50	11	0.5
4	100	12	2
5	500	13	5
6	100	14	10
7	2000	15	50
8	6960	16	84

3. Results

Results on cyanobacteria density expressed as average cell variation in the experiment where phosphorus concentration varied are presented in Figure 1. The coefficient of variation of the results was always less than 10%. After 24 hours of incubation, the growth was relatively low for the two strains and appeared similar under all phosphorus concentrations. In P-sufficient cultures with an initial phosphate concentration of 1000–6960 μg l^{-1}, a 5–7 day period of exponential growth was observed. The maximum density was 470–900 $\times$ 10^5 cells ml^{-1} and 260–300 $\times$ 10^3 trichomes ml^{-1}, for *M. ichthyoblabe* and *A. aphanizomenoides*, respectively. After this period, a stationary phase lasted until the tenth day. However, in P-free and in P-deficiency media (0–500 μg l^{-1}), the growth was limited and did not exceed 225 $\times$ 10^5 cells ml^{-1} and 92 $\times$ 10^3 trichomes ml^{-1}, respectively, for *M. ichthyoblabe* and *A. aphanizomenoides*. Between days 5 and 6, the cyanobacteria were able to maintain those density levels, but after that cell density quickly decreased and cells turned yellowish. In the case of *A. aphanizomenoides* cultures, the microscopic observations revealed the onset of akinete development in many filaments starting between days 5 and 6.

The analysis of data at different nitrogen concentrations showed that only *M. ichthyoblabe* (Figure 2A) presented growth responses similar to those obtained for the phosphorus test. The highest cell density of *Microcystis* (790–935 $\times 10^5$ cells ml^{-1}) was observed in the highest nitrogen concentrations of 50 and 84 mg l^{-1}, after an exponential growth phase of approximately 8 days. In the cultures grown at the lowest concentrations (0–10 mg N l^{-1}), the growth ceased after the first two incubation days and the cellular densities remained relatively stable during a stationary phase until the fourth day. After this, the density decreased gradually and the cultures lost their initial blue-green colour, as in the case of P-deficient cultures. In opposition to what happened with *M. ichthyoblabe*, the nitrate-nitrogen concentration did not have a large effect on the growth of *A. aphanizomenoides* (Figure 2B). *Anabaena* cultures grew in a similar way to that of *Microcystis* and reached high densities of 230–260 trichomes ml^{-1} during an exponential growth of 8–9 days in the media with or without nitrate. Morphological differences between *Anabaena* trichomes in different treatments were observed from the second day of incubation. An increased number of heterocysts ($\sim$3–4/trichome) in the media without nitrate-nitrogen or in the low nitrate concentration media (i.e. 0.05–5 mg l^{-1}) were observed. The trichomes growing in the higher concentration (10–50 mg l^{-1}) did not develop heterocysts, as in the normal Z8 media (84 mg l^{-1}).

Major differences were observed in the growth response of both *M. ichthyoblabe* and *A. aphanizomenoides* growing under different N:P supply ratios (Figure 3). For *Microcystis*, N:P ratios of 12 (Z8) and 15 seemed to be optimal for growth, since cell densities were much higher (700–800 $\times 10^5$ cells ml^{-1}) than those recorded in all other cultures. This cyanobacterium exhibited a limited growth and cell densities rapidly decreased after 4–5 days under very low (1, 5) or

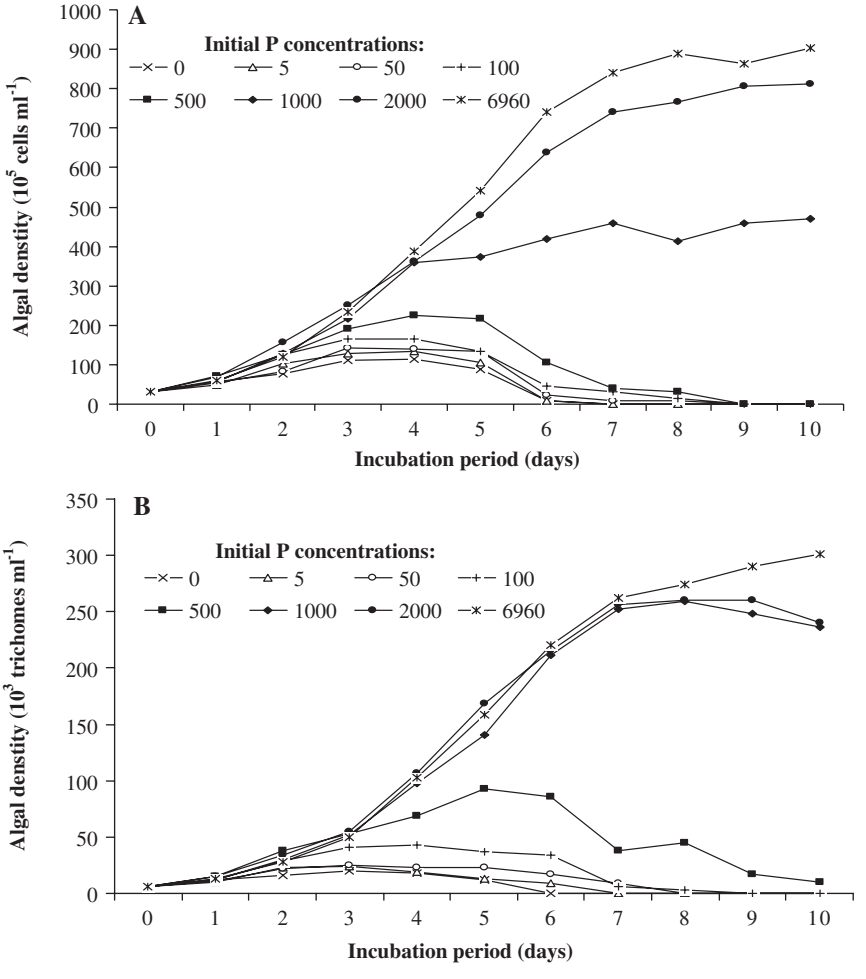

Figure 1. Effect of phosphorus concentrations on the growth of *Microcystis ichthyoblabe* (A) and *Anabaena aphanizomenoides* strains (B).

very high (30, 100, 500) N:P ratios, which corresponded respectively to a potential nitrogen or phosphorus limitation. A different trend was observed in the *A. aphanizomenoides* cultures, since cell density strongly increased during the incubation period under N:P ratios from 1 to 15. They seemed not to be limited under potential nitrate-nitrogen limitation (N:P of 1 and 5), unlike the *Microcystis* cultures. However, high N:P (30–500) supply ratios markedly influenced growth of *Anabaena*, where a gradual decrease in cell densities was noted from the sixth incubation day.

4. Discussion

Because cyanobacterial blooms often develop in eutrophic lakes, it was originally assumed that they required high phosphorus concentrations. This assumption was maintained even though cyanobacterial blooms often occurred when concentrations of dissolved phosphate were very low [12]. In the Oued Mellah lake, phosphorus has also been pointed out as a limiting nutrient for algal growth during summer, when *Microcystis ichthyoblabe* and *Anabaena aphanizomenoides*

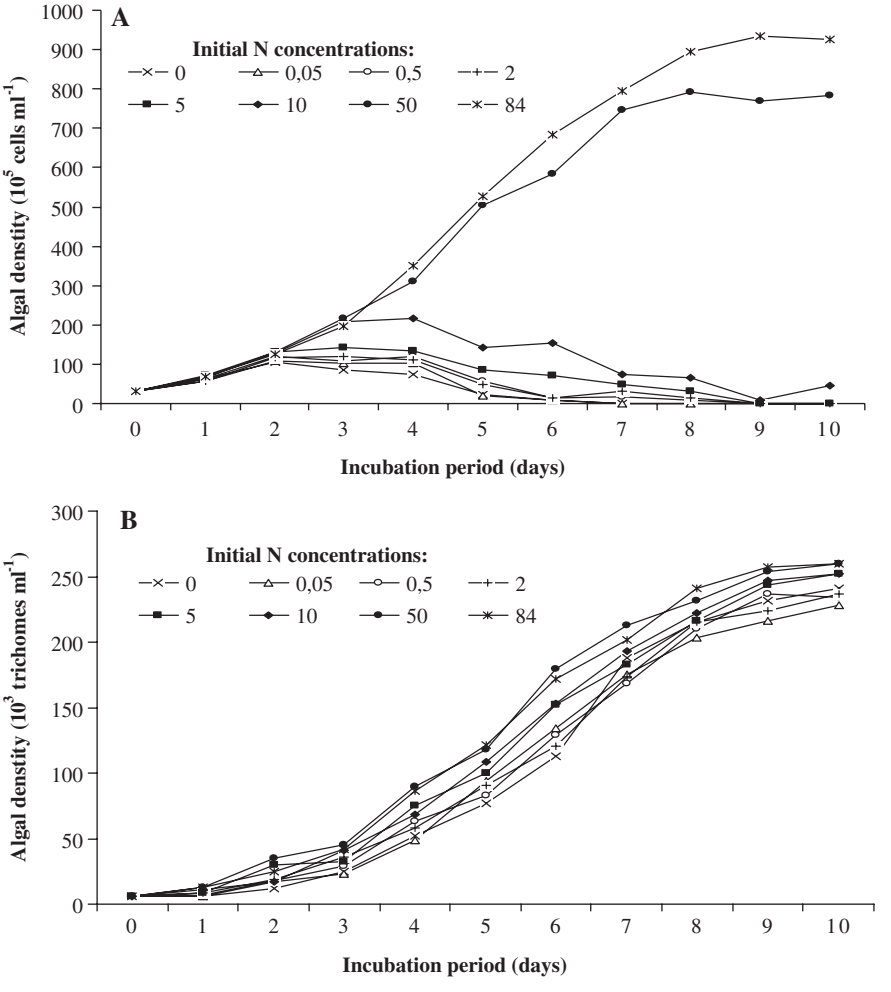

Figure 2. Effect of nitrate-nitrogen concentrations on the growth of *Microcystis ichthyoblabe* (A) and *Anabaena aphanizomenoides* strains (B).

blooms occurred [4,5]. The growth of strains of these two species was investigated under sub-optimal light and temperature conditions. With this experiment, we confirmed that phosphorus concentration had an important effect on their growth. With high nutrient concentrations, strains grew better than with P-deficiency. In spite of phosphorus deficiency in the treatment without additional phosphorus or with very low initial P concentrations, both *M. ichthyoblabe* and *A. aphanizomenoides* maintained their growth for five to six days. This result indicates that the growth was supported by the intracellular phosphorus stored within cells before the experiments. Other species of cyanobacteria also have a substantial storage capacity for phosphorus. This enables them to perform up to two to four cell divisions corresponding to a 4–32 fold increase in biomass, even if no soluble reactive phosphorus is measured [12].

The observed akinete apparition under phosphorus limitation could be considered as a stress response to P-deficiency. Akinetes are often produced in large numbers by senescent populations. In general, conditions inhibiting continued cyanobacterial growth promote differentiation of vegetative cells to akinetes. We may include: (a) deficiency in inorganic nitrogen supply; (b) depletion of available phosphorus; (c) deficiency in iron; and (d) light limitation [13]. In *Anabaena*,

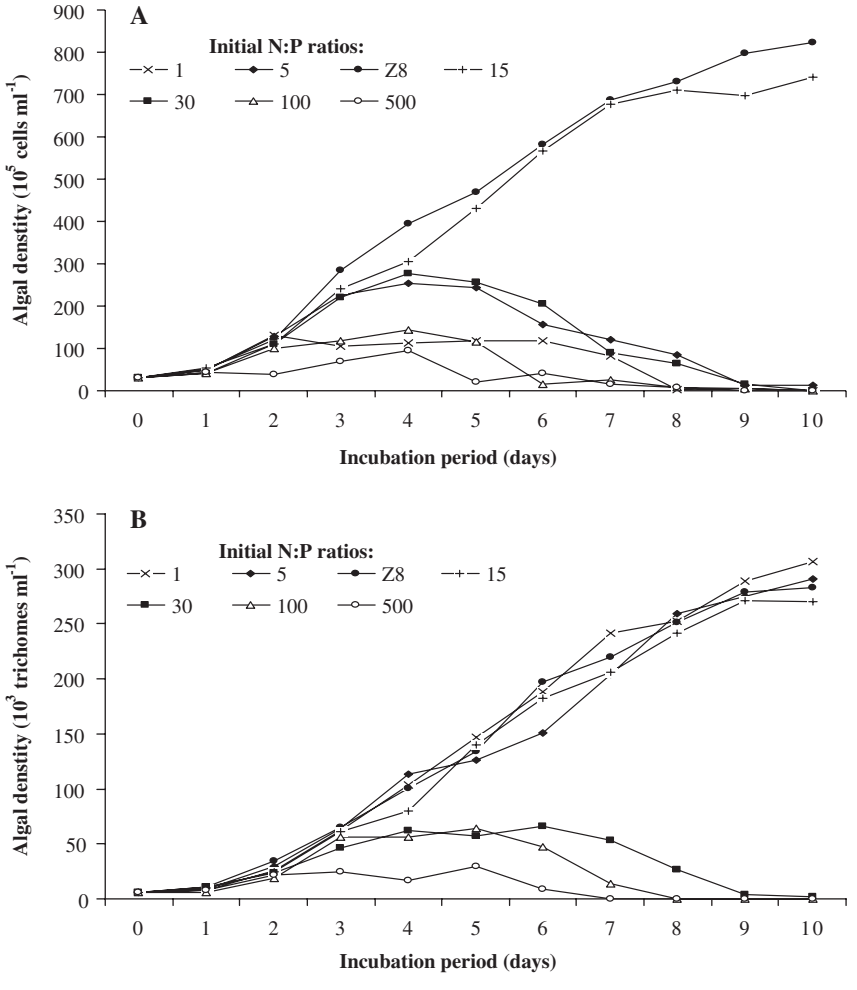

Figure 3. Growth kinetic of *Microcystis ichthyoblabe* (A) and *Anabaena aphanizomenoides* strains (B) under different N:P ratios.

phosphate deficiency has been shown to be the most significant factor controlling the development of akinetes [14].

As with phosphorus experiments, cell growth of *M. ichthyoblabe* was substantially favoured under high nitrate concentrations, whereas cultures under N-free or N-deficient conditions seemed to be limited. It seems that *Microcystis* cells must use their reserves to survive under nitrogen limitation. This corroborates with the result of Sbiyyaa et al. [15], who showed that internal nitrogen quotas allow four days of growth for *Microcystis* and *Synechocystis* under nitrogen deficiency. This is also in agreement with field data recorded in the Oued Mellah lake, where nitrate-nitrogen was very low during *M. ichthyoblabe* blooms in 1997 and 1999 (0–0.8 mg $N\text{-}NO_3$ l^{-1}, [4]). Nitrate-nitrogen limitation was still pronounced in late summer (0–0.18 mg $N\text{-}NO_3$ l^{-1}, [5]) and would be unlikely to limit the growth of cyanobacteria species, since the heterocystous *A. aphanizomenoides* proliferated and formed heavy water-blooms. This species has the additional ability to assimilate atmospheric nitrogen. In fact, the low level of dissolved nitrate-nitrogen could be competitively advantageous to the diazotrophic cyanobacteria because it would limit the growth of other algae [16]. Heterocystous cyanobacteria may be able to increase their biomass in

Table 2. Optimum N:P ratios by weight for various cyanobacteria.

Cyanobacteria	N:P ratios	Reference
Microcystis sp.	4	[20]
Microcystis aeruginosa	8.5	[21]
Microcystis aeruginosa	18	[22]
Microcystis ichthyoblabe	12–15	This study
Anabaena flos-aquae	< 8	[23]
Anabaena aphanizomenoides	1–15	This study
Pseudanabaena catenata	9	[24]
Oscillatoria aghardii	12	[25]
Oscillatoria aghardii	21	[22]
Synechococcus linearis	11–20	[26]
Synechococcus sp.	20	[27]

response to pulses of P even at low concentrations [8]. In an experiment using P at levels similar to our maximum P level provided in experiments 9–16, Spróber et al. [6] found that the biomass of the heterocystous *C. raciborskii* was not affected by the amount and forms of N supplied.

The N:P ratio is among the major discussed factors favouring cyanobacterial dominance in a variety of lakes [17]. This study revealed differences in the growth of *M. ichthyoblabe* and *A. aphanizomenoides* as a function of N:P variation. A markedly diminished growth was observed with the lower N:P ratios (≤ 5) only for *Microcystis* and with the highest N:P ratios (≥ 30) for both *Microcystis* and *Anabaena*. This confirms that cells must have used their reserves to survive for a few incubation days under nutrient limitation, and after that a process of cellular imbalance took place. *Anabaena*, however, supported nitrate-nitrogen limitation and showed an optimal growth under N:P ratios from 1 to 15. For *Microcystis* only, ratios of 12 (Z8 medium) and 15 seemed to be optimum. The laboratory studies of Rhee [18] and Tilman et al. [19] have shown that individual species have optimum nutrient ratios, which act as transitional points where growth limitation is transferred from one nutrient limitation to another. In the case of cyanobacteria, optimum N:P ratios (Table 2) are highly variable even for one genus (e.g. *Synechococcus*) or species (e.g. *Microcystis aeruginosa*, *Oscillatoria aghardii*), but remained relatively low. A combination of elevated temperature and high P concentration may lead not only to increased *Microcystis* blooms but also to an increase on toxic strains versus non toxic [9]. This may lead to an increased toxin content of blooms and increased human and environmental health risks.

Further ecophysiological studies are needed to evaluate the role of other important factors, such as carbon dioxide, different P and N sources, micronutrients, light and temperature – nutrient interactions. The insights gained from the laboratory experiments should be tested under field conditions before further conclusions are made. Understanding the regulatory factors will lead to the development of means to control harmful blooms of cyanobacteria.

Acknowledgements

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