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Variation of Microcystins, Cyanobacterial Hepatotoxins, in *Anabaena* spp. as a Function of Growth Stimuli

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Cyanobacterial hepatotoxins, microcystins, are specific inhibitors of serine/threonine protein phosphatases and potent tumor promoters. They have caused several poisonings of animals and also pose a health hazard for humans through the use of water for drinking and recreation. Different strains of the same cyanobacterial species may variously be nontoxic, be neurotoxic, or produce several microcystin variants. It is poorly understood how the amount of toxins varies in a single strain. This laboratory study shows the importance of external growth stimuli in regulating the levels and relative proportions of different microcystin variants in two strains of filamentous, nitrogen-fixing *Anabaena* spp. The concentration of the toxins in the cells increased with phosphorus. High temperatures (25 to 30°C), together with the highest levels of light studied (test range, 2 to 100 μmol m^{-2} s^{-1}), decreased their amount. Different structural variants of microcystins responded differently to growth stimuli. Variants of microcystin (MCYST)-LR correlated with temperatures below 25°C, and those of MCYST-RR correlated with higher temperatures. Nitrogen added into the growth medium and increasing temperatures increased the proportion of microcystin variants demethylated in amino acid 3. All variants remained mostly intracellular. Time was the most important factor causing the release of the toxins into the growth medium. Time, nitrogen added into the growth medium, and light fluxes above 25 μmol m^{-2} s^{-1} significantly increased the concentrations of the dissolved toxins. According to the results, it thus seems that the reduction of phosphorus loads in bodies of water might play a role in preventing the health hazards that toxic cyanobacterial water blooms pose, not only by decreasing the cyanobacteria but also by decreasing their toxin content.

Strains of several cyanobacterial genera, *Microcystis*, *Anabaena*, *Oscillatoria*, and *Nostoc*, produce high levels of hepatotoxins, microcystins (4, 15, 26, 31, 32, 40). These specific inhibitors of serine/threonine protein phosphatases (11, 12, 18) are potent tumor promoters (21) with the general structure of cyclo(D-Ala^1–X^2–D-$MeAsp^3$–Z^4–$Adda^5$–D-Glu^6–$Mdha^7$), in which X and Z are variable L amino acids, D-MeAsp is D-*erythro*-β-methylaspartic acid, Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, and Mdha is *N*-methyldehydroalanine (5). At present, nearly 50 microcystin variants are known with variation in amino acids 2 and 4, methylation in amino acids 3 and/or 7, modification of Adda, and changes in amino acids 1, 6, or 7 (26, 31). Their acute toxicity (given intraperitoneally to mice) varies between 50 and 800 μg/kg of body weight (26). Microcystins have caused several poisonings of domestic animals and wildlife around the world, and they also pose a health hazard for humans through the use of water for drinking and recreation (25).

In bodies of water, the most recognized causative agents for cyanobacterial mass occurrences are eutrophication, warm water temperatures, high light intensity, and stable weather conditions (7, 22, 25). Surveys of these water blooms have shown that 25 to 95% of them are toxic (1, 22, 24, 34). However, it is poorly understood what causes a water bloom to be toxic, or how the toxin content of a single strain varies. *Anabaena* strains coexisting in the water blooms are known either to produce hepatotoxins or neurotoxins or to be nontoxic (9, 34), and microcystins produced by these strains show a high degree of structural variation (32, 33). While no study on the variation of microcystin content in *Anabaena* has been published, several previous ones with other toxic cyanobacterial genera have been hampered by the fact that cyanobacteria produce several microcystin variants and by the lack of methods to quantify them (for reviews, see references 25, 31, and 40). Characterization of a variety of microcystins in *Anabaena* strains (33) and the development of methods for quantifying them made it possible to investigate to a broader extent than previously the variation in and relative proportions of microcystins in these strains under different growth conditions.

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MATERIALS AND METHODS

Organisms and culturing. Two *Anabaena* sp. strains, 90 and 202A1, isolated in 1986 and 1987, respectively, from cyanobacterial water blooms at Lake Vesijärvi, Southern Finland, and purified until axenic (27), were used in this study. Together, they have been shown to produce ten different microcystin variants (32, 33). To study the effects of temperature, light, phosphorus, and nitrogen on growth, toxin levels, and relative abundance of microcystins in the strains and in their growth media, five experiments were conducted in batch cultures in 5-liter Erlenmeyer flasks with 3 liters of growth medium (for test ranges, see Table 1). The growth medium was Z8 minus nitrogen (13). In the nutrient experiments, the concentration of orthophosphate was reduced by replacing K_2HPO_4 with KCl so that potassium was unchanged, and the concentration of nitrate was increased by adding an appropriate amount of $NaNO_3$. The flasks were incubated with continuous stirring with sterile air in temperature-controlled water baths. Continuous cool white fluorescent irradiance (Daylight Deluxe tubes; Airam, Helsinki, Finland) incident on the surface of the culture flasks was measured with an LI-1000 datalogger photometer (Li-Cor, Inc.; Lincoln, Nebr.). Before the phosphorus experiment, the strains were grown for 1 week in phosphorus-free medium to deplete cells of internal stores of phosphorus. The inocula were grown for 7 days, and each inoculum was 100 ml per 3 liters of growth medium.

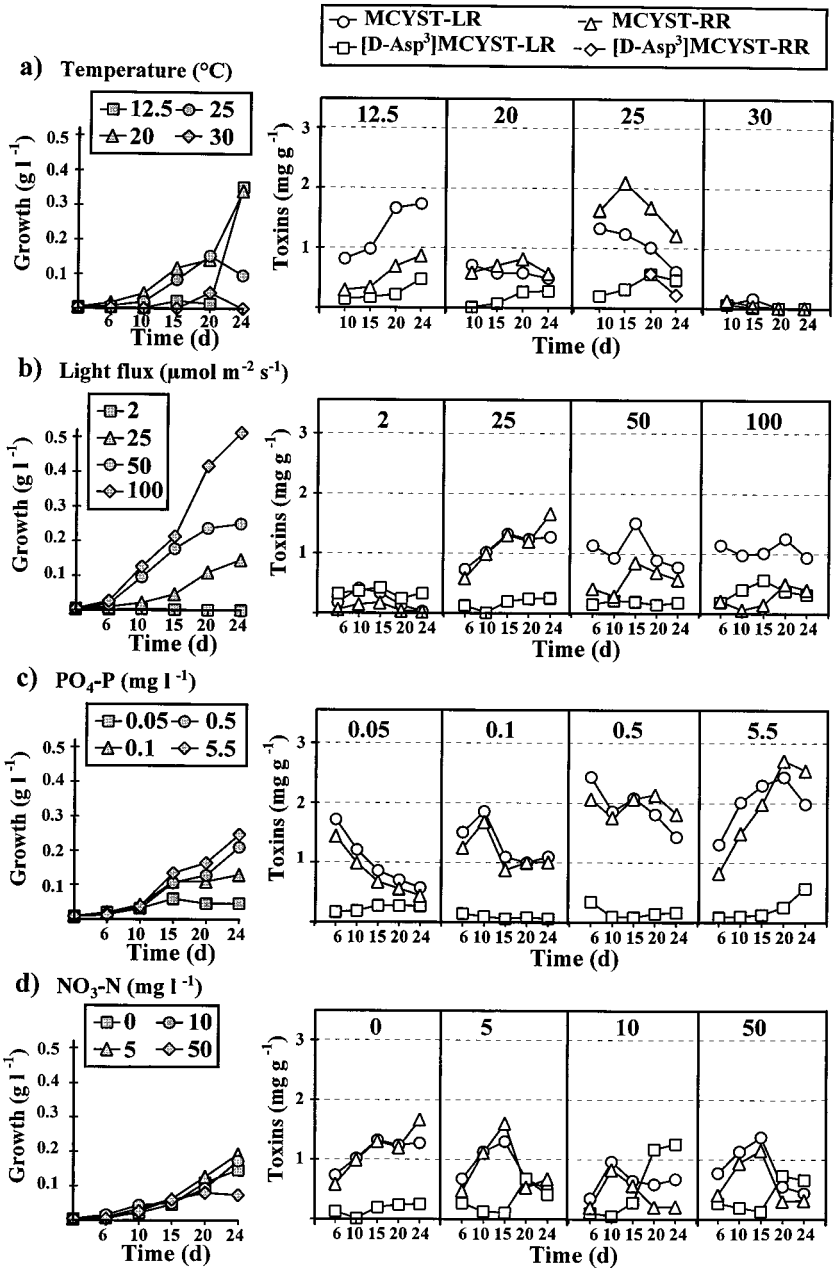

FIG. 1. Effects of temperature (a), light flux (b), PO_4-phosphorus (c), and NO_3-nitrogen (d) on growth (left) and microcystin content in the cells (right) of *Anabaena* sp. strain 90.

Growth and toxin analysis. Growth was followed by measuring of dry weights at each sampling day by filtering 50 ml of the culture to tared GF52 glass-fiber filters (Schleicher & Schuell, Dassel, Germany), which were then dried for 24 h at 100°C. For toxin analysis, the cells were harvested by centrifugation at 12,000 × *g* at 20°C, frozen, and stored at −20°C until analyzed (1 to 4 weeks). From the freeze-dried cell material, the toxins were extracted with water by sonicating, and the samples were prepared as previously described (19). Toxins dissolved in the growth medium were analyzed from the supernatant. One hundred milliliters of the supernatant was filtered through glass-microfiber filters and concentrated on octyldecyl silane cartridges (Bond Elut C_{18}; Varian) (10). The cartridges were washed with 5 ml of 20% methanol prior to elution with 10 ml of methanol. The samples were then dried in an airstream and dissolved in 500 μl of 20% methanol. Microcystins in cell material and those dissolved in growth medium were analyzed with a Hewlett-Packard HP1090 liquid chromatograph equipped with a Hewlett-Packard UV/Vis diode array detector. A Regis Pinkerton GFF-S5-80 (4.6 by 150 mm) internal surface reverse-phase column was used, and the mobile phase was as follows: 0 to 5 min, 15% acetonitrile–85% 0.1 M phosphate buffer (pH 6.5); 5 to 7 min, linear gradient of 15 to 20% acetonitrile; and 7 to 18 min, 20% acetonitrile. The toxins were identified by their UV spectra and retention times and by spiking the samples with purified standards of MCYST-LR, MCYST-RR, their variants demethylated in amino acids 3 and 7, and variants didemethylated in both of these amino acids. In addition, the toxin peaks were isolated and identified according to their mass spectra. The concentrations of the toxins were determined by extrapolating peak areas to a calibration curve ($n = 4$; $r^2 = 0.989$) determined with purified [D-Asp^3]MCYST-RR as a standard. The detection limit for the microcystins in cell material was 0.01 mg g^{-1}, and for those released into the growth medium, it was 0.5 μg $liter^{-1}$.

Statistical analyses. The analyses were performed with MATLAB statistical software for Windows (MathWorks, Inc., Natick, Mass.). Composite design matrices were created with prior transformation of original variables into coded ones. The matrices were expanded to include the coeffect factors of test variables by multiplying the columns of the coded variables by each other and the qua-

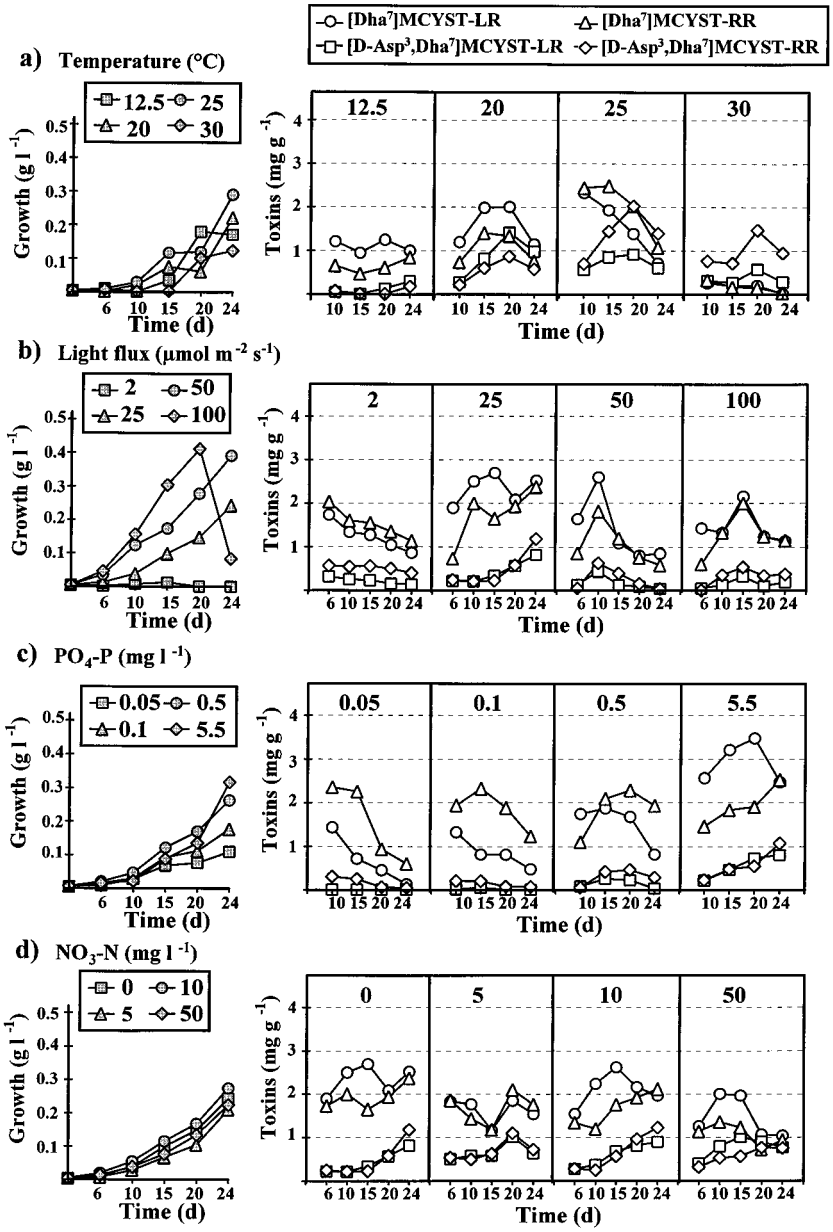

FIG. 2. Effects of temperature (a), light flux (b), PO_4-phosphorus (c), and NO_3-nitrogen (d) on growth (left) and microcystin content in the cells (right) of *Anabaena* sp. strain 202A1.

dratic factors by multiplying them by themselves. Multivariate regression analysis was then performed. For the analyses, the values for toxin concentration (mg g^{-1} of freeze-dried cells) and light flux (μmol m^{-2} s^{-1}) were ln-transformed in order to decrease heteroscedasticity of the variances. Multivariate regression models to describe the variation of toxins were obtained by omitting insignificant factors.

RESULTS

Toxin variants. *Anabaena* strain 90 produced MCYST-LR and MCYST-RR as main toxins and their variants demethylated in amino acid 3 as minor toxins. Of these, [D-Asp^3]MCYST-LR was detected throughout the experiments in low concentrations, and [D-Asp^3]MCYST-RR was detected occasionally, only at high temperatures. In strain 202A1, microcystins demethylated in amino acid 7, [Dha^7]MCYST-LR and [Dha^7]MCYST-RR, were in most cases the main toxins. Their variants demethylated also in amino acid 3, e.g., [D-Asp^3, Dha^7]MCYST-LR and [D-Asp^3,Dha^7]MCYST-RR, were generally detected in lower concentrations. The other toxins that the strains have previously been shown to produce were not detected with high-pressure liquid chromatography due to their low amounts.

Temperature. The overall toxin pool of both the strains was highest at 25°C (Fig. 1a and 2a). Lower and higher temperatures decreased their amounts (Table 1 shows negative regression coefficients for the quadratic factor of temperature). MCYST-LR in strain 90 and [Dha^7]MCYST-LR in strain 202A1 correlated with temperatures below 25°C, and the corresponding variants of MCYST-RR correlated with higher temperatures. A rise in temperature increased the relative proportion of toxins demethylated in amino acid 3. The different behaviors of the toxins can be seen also from the regression

TABLE 1. Significant regression coefficients of microcystins in the cells of *Anabaena* in response to growth stimuli[a]

Expt no. and variable: test range	Regression coefficient for:					
	Anabaena sp. strain 90		*Anabaena* sp. strain 202A1			
	MCYST-LR	MCYST-RR	[Dha^7] MCYST-LR	[Dha^7] MCYST-RR	[D-Asp^3,Dha^7] MCYST-LR	[D-Asp^3,Dha^7] MCYST-RR
1						
Temp: 12.5–30°C	−1.4830***	−1.0414**	−0.9669***	−0.5715*	0.7979**	1.6934***
(Temp)^2	−1.5018*	−2.3066**	−1.6483***	−1.7280***	−1.6384**	−1.4044*
2						
Light: 2–100 μmol m^{-2} s^{-1}	0.9091***	0.4938*				
(Light)^2	−0.8271**	−2.0512***	−0.4056*			
3						
P: 0.05–5.5 mg liter^{-1}	0.3149***	0.3530***	0.7817***		1.9450***	0.6949**
Time × P	0.2785**	0.4311***	0.3497*		0.5218*	
P^2	−1.8308***	−2.5603***	−2.6297**		−7.2838***	
4						
N: 0–50 mg liter^{-1}		−0.4042*	−0.2031*	−0.2886***	0.2886**	
N^2	0.7964**	1.5532**				
5						
N: 0, 84 mg liter^{-1}	−0.5247***	−0.8334***		−0.3155*		
Light: 2, 25, 100 μmol m^{-2} s^{-1}		−1.3376***	−0.6340**	−1.3627***	−1.2625***	−1.7786***
Temp: 15, 25°C			−0.9326***			0.4594**
Time × temp			−0.7709***	−0.5590**	−0.3571*	−0.5561*
Temp × light		−0.3886*	−0.7122***	−0.3806*	−0.8184***	−0.8235***

[a] Coefficients are given for coded values of variables. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

coefficients (Table 1): toxins demethylated in amino acid 3 increased (positive regression coefficients), and the other toxins decreased (negative regression coefficients), with temperature.

Light. Induced by light, the level of MCYST-LR in strain 90 was saturated at a relatively low light level (2 to 25 μmol m^{-2} s^{-1}) and remained constant at levels higher than this (Fig. 1b). Due to the positive regression coefficient of the linear and the negative regression coefficient of the quadratic effect of light (Table 1), the concentration of MCYST-LR as a function of light is a wide downward-opening parabola. With a less significant linear and highly significant quadratic factor of light, the optimum light flux for the concentration of MCYST-RR is sharper: its level is clearly highest at 25 μmol m^{-2} s^{-1}. Light had no effect on the toxins of strain 202A1 at 20°C (Fig. 2b and Table 1). When the strain was grown at lower (15°C) and higher (25°C) temperatures, it was shown that temperature and light had a coeffect on toxin concentrations (Table 1, experiment 5). The negative regression coefficients for the coeffect of light and temperature mean that at 15°C the amounts of toxins increased and that at 25°C their amounts decreased as a function of light.

Phosphorus. In addition to growth, the intracellular toxin levels increased with phosphorus (Fig. 1c and 2c; Table 1). The significant positive coeffect of time and phosphorus means that during the time course, the toxin pool decreased in the cells grown under phosphorus limitation and increased in the cells grown at high phosphate concentrations.

Nitrogen. Nitrate-nitrogen concentration had no significant effect on the growth of the strains. Toxins demethylated in amino acid 3 increased (Fig. 1d and 2d), and the levels of their methylated variants decreased (Table 1), when nitrate-nitrogen was added into the growth medium.

Toxins in growth media. Compared to the levels of the intracellular microcystins, the concentrations of the toxins detected dissolved in the growth medium were low, and in most cases the variants were present in the same proportions as were detected in the cell material. Time was the most significant factor causing the release of the toxins, but light (Fig. 3) and nitrogen (Fig. 4) also increased it. Although temperature decreased and phosphorus increased the intracellular levels of toxins, they had no effect on the extracellular levels (data not shown).

Mathematical modelling. The results of the multivariate regression analyses were formulated into mathematical equations by omitting the insignificant factors ($P > 0.05$). The external phosphate-phosphorus concentration proved to produce models with the highest coefficients of determination (Fig. 5). In the other experiments, models with only significant factors proved to be insignificant ($r^2 < 0.70$) for most of the toxins.

DISCUSSION

This study revealed the importance of external growth stimuli in regulating the levels and relative proportions of microcystins in the filamentous, nitrogen-fixing cyanobacterium *Anabaena*. It also proved that a statistical approach allowing mathematical modelling, previously ignored in the studies of cyanobacterial toxicity, is an efficient tool in the description of the complex variation of microcystins in the cells of cyanobacteria.

The results show that not only the growth of *Anabaena* but also the levels of intracellular hepatotoxins increased with phosphorus. Previously, growth-limiting phosphorus concentrations have been shown to decrease the content of micro-

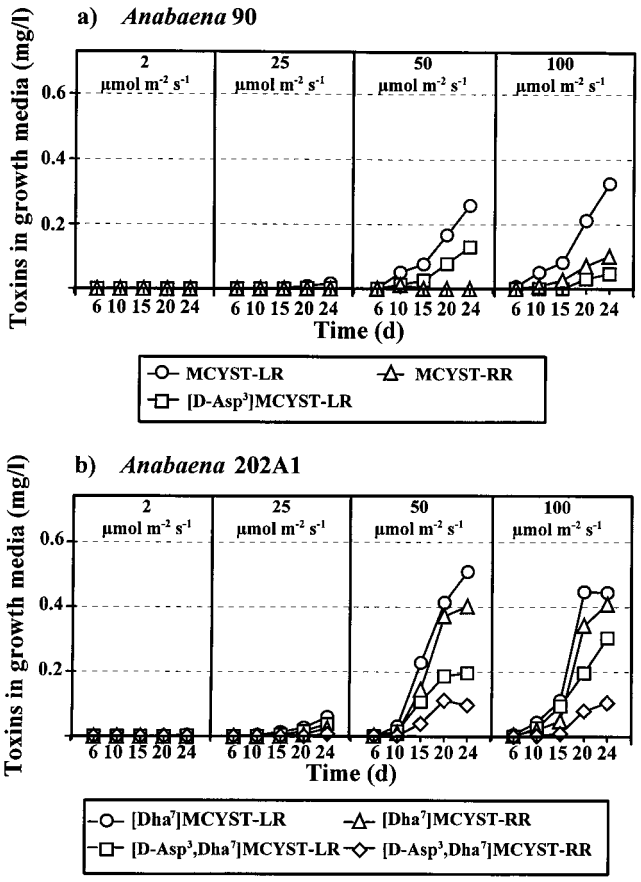

FIG. 3. Effect of light on the concentration of microcystins from *Anabaena* sp. strain 90 (a) and *Anabaena* sp. strain 202A1 (b) detected dissolved in the growth medium.

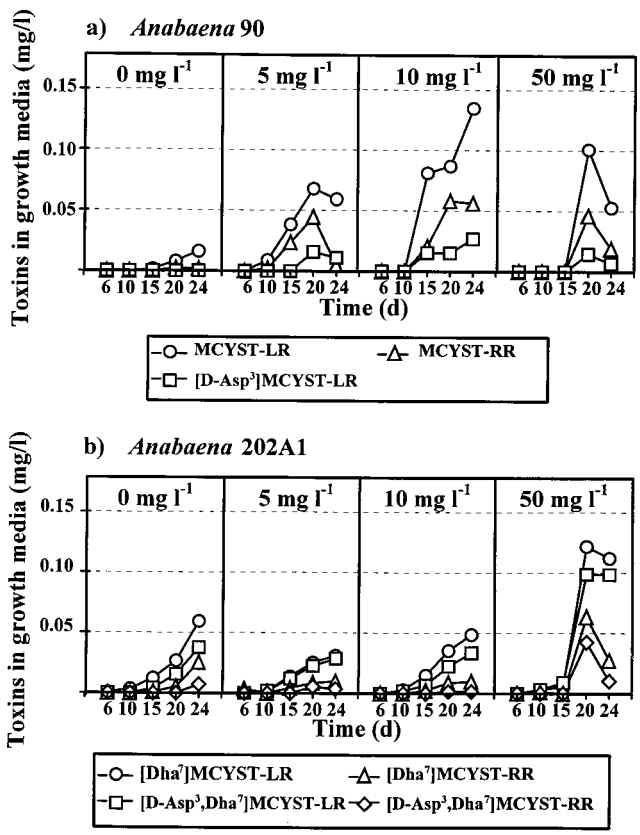

FIG. 4. Effect of NO_3-nitrogen on the concentration of microcystins from *Anabaena* sp. strain 90 (a) and *Anabaena* sp. strain 202A1 (b) detected dissolved in the growth medium.

cystins in filamentous non-nitrogen-fixing *Oscillatoria agardhii* strains (30). Reduction of phosphorus slightly decreased (41), and its removal from the growth medium did not influence (6), the toxicity of unicellular non-nitrogen-fixing *Microcystis aeruginosa* as measured by mouse bioassay. Field studies have indicated a positive correlation of total phosphorus with MCYST-LR in cells of *M. aeruginosa* (14) or in particulate phytoplankton material consisting predominantly of *Microcystis* spp. (16). This suggests that microcystins in different cyanobacterial genera respond in a similar way to the extracellular phosphorus concentration: not only cyanobacterial growth but also the amounts of intracellular hepatotoxins increase with phosphorus.

While the two *Anabaena* strains in this study showed the highest levels of microcystins in a nitrogen-free growth medium, decreased toxicity upon removal (6) or reduction (37, 41) of nitrate from the medium was reported for *M. aeruginosa*, and high toxin levels in *Oscillatoria* correlated with high nitrogen concentrations (30). Thus, nitrogen-fixing and non-nitrogen-fixing cyanobacteria seem to differ in their responses to external nitrogen concentration.

According to this and previous studies, high and low temperatures compared to the optimal temperature for the strain decrease the toxicity or concentrations of microcystins (6, 8, 28, 30, 38). This study revealed a clear difference between the variants of MCYST-LR and MCYST-RR, the former being detected more at lower and the latter more at higher temperatures. Furthermore, rising temperatures increased the concentrations of microcystin variants that are demethylated in amino acid 3.

Controversial results have been obtained for the effect of light. Early studies revealed no great differences in toxicity for *Microcystis* grown under different irradiances (6, 8), but several studies show that low levels of light decrease toxin levels (36, 38, 41). Perhaps not enough attention has been paid to the spectral quality of the light used, and due to different temperature and light optima of the strains, the coeffect of light with temperature should be taken into consideration. In this study, the strains differed in that *Anabaena* sp. strain 202A1 showed a preference for lower light levels and higher temperatures than strain 90. Under certain conditions (30°C for strain 90 and 2 μmol m^{-2} s^{-1} for both the strains), essentially no changes in cell number occurred. The viability of the cells was checked by microscopic examination, but further tests of the viability of the cultures under these conditions were not undertaken. Light levels tested had no significant effect on the toxins of strain 202A1, showing that its toxin concentrations were saturated at low light, as was the MCYST-LR concentration of strain 90 at irradiances of 2 to 25 μmol m^{-2} s^{-1}. For MCYST-RR strain 90 had a sharp, relatively low optimum level of light, 25 μmol m^{-2} s^{-1}. Since both of the strains grew better the higher the light flux, microcystin contents of the cells growing at different light fluxes did not correlate positively with growth, unlike the response to phosphorus or temperature. *Anabaena* preferred higher levels of light than the previously studied *Oscillatoria* (30). At a high temperature (25°C), increasing the irradiance

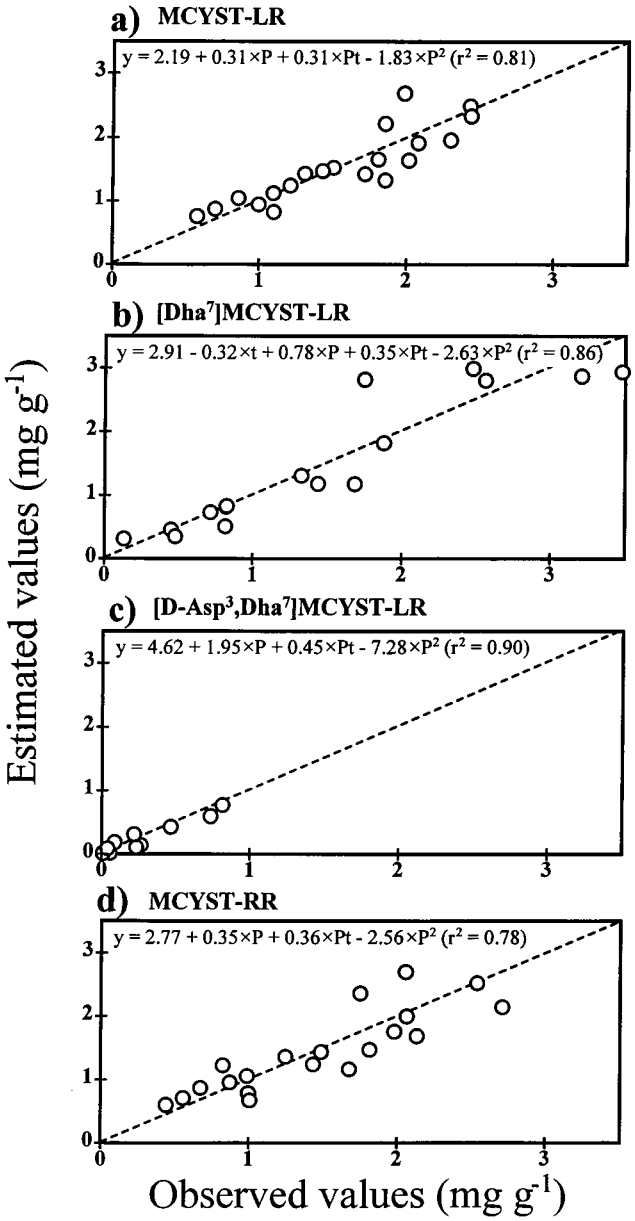

FIG. 5. Mathematical models for the effect of PO_4-phosphorus on the levels of different microcystin variants in the cells of the two strains studied. Values estimated by models are plotted against observed values. Regression coefficients are given for the coded values of variables: t, time (in days); P, concentration of PO_4-P in the growth medium (mg liter^{-1}); Pt, coeffect of phosphorus and time; P^2, quadratic factor of phosphorus. y, ln-transformed toxin level (mg g^{-1} of freeze-dried cells); r^2, coefficient of determination for the model. Only models with r^2 values of >0.70 for the model and P values of <0.05 for each factor are presented.

decreased the amounts of microcystins produced by both genera. Thus, in general, it seems that microcystin levels in cyanobacterial cells are saturated at low light, remain relatively constant at a large range of irradiance, and decrease as a coeffect of high temperature and high light. Evidence from this study, however, indicates that not all microcystin variants respond in a similar way.

In the two *Anabaena* strains, variants of MCYST-LR and MCYST-RR responded differently to growth stimuli: high temperature and nitrogen added into the growth medium increased the proportion of toxins demethylated in amino acid 3 compared with their corresponding methylated variants. In both the strains, variants of MCYST-LR correlated with lower temperatures than those of MCYST-RR and the overall intracellular toxin content was highest at 25°C. In strain 90, MCYST-LR correlated with higher and [D-Asp^3]MCYST-LR with lower levels of light. Unlike the other growth factors, increasing phosphorus concentrations caused increases in the levels of all the toxins. Previously, it has been shown that in *M. aeruginosa* temperature and, to a lesser extent, fluence rate had marked effects on the relative proportions of three toxic peptides identified from the strain (39) and that the relative concentrations of MCYST-LR, MCYST-RR, and MCYST-YR may change with time (42).

Compared to neurotoxic—anatoxin-a-producing—*Anabaena* strains previously studied in our laboratory, the most pronounced differences were the responses of the hepatotoxic strains to light and to phosphorus. At high light levels, hepatotoxic strains grew better than neurotoxic strains (23), and their toxin levels did not decrease as did the levels of anatoxin-a. Lack of phosphorus had no effect on anatoxin-a levels (23) but decreased the amount of hepatotoxins produced.

It seems that release of the microcystins is mostly due to cell death or leakiness, since the concentrations detected in the growth medium were low compared to intracellular levels, time was the most important factor causing the release of the toxins, and the concentrations of different variants in the medium were proportional to their concentrations in the cells. Similar results have been obtained with other hepatotoxic cyanobacteria (2, 17, 30) and in field studies (16, 35, 43).

The role of toxins in cyanobacteria is still not understood. Microcystins, potent inhibitors of serine/threonine protein phosphatases, have been suggested to act as protective compounds against grazing by zooplankton (4) or as intracellular chelators inactivating free cellular Fe^{2+} (37) or to have some specific cell regulatory function (29). Recently, it was shown that protein phosphatase activity in a marine dinoflagellate, *Prorocentrum lima*, appears to be exquisitely regulated by ocadaic acid, a well-known inhibitor of protein phosphatases produced by the organism (3). Trying to identify similar relationships between cyanobacterial protein phosphatases and microcystins would be tempting, especially since the concentrations of microcystins in cyanobacterial cells are similar to those of ocadaic acid in *P. lima* (20). Revealing the variation of microcystins as a function of growth stimuli may give us hints of their role and may help us to understand the reasons for the high variability in toxicity of cyanobacterial water blooms.

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