J. Phycol. **47,** 200–207 (2011)

DOI: 10.1111/j.1529-8817.2010.00931.x

THE TOXIC CYANOBACTERIUM *NOSTOC* SP. STRAIN 152 PRODUCES HIGHEST AMOUNTS OF MICROCYSTIN AND NOSTOPHYCIN UNDER STRESS CONDITIONS[1]

Rainer Kurmayer[2]

Austrian Academy of Sciences, Institute for Limnology, Mondseestrasse 9, 5310 Mondsee, Austria

The understanding of how environmental factors regulate toxic secondary metabolite production in cyanobacteria is important to guarantee water quality. Very little is known on the regulation of toxic secondary metabolite production in benthic cyanobacteria. In this study, the physiological regulation of the production of the toxic heptapeptide microcystin (MC) and the nontoxic related peptide nostophycin (NP) in the benthic cyanobacterium *Nostoc* sp. strain 152 was studied under contrasting environmental conditions. A 2^k levels factorial design was used, where k is the number of four factors that have been tested: reduction in temperature (20°C vs. 12°C), irradiance (50 vs. 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$), P-$PO_4$ (144 vs. 0.14 µM P-PO_4), and N-NO_3 (5.88 mM versus N-NO_3 free). While the growth rate was reduced >100-fold under most severe conditions of temperature, irradiance, and phosphate reduction, the production of MC and NP never ceased. The MC and NP contents per cell varied at maximum 5- and 10.6-fold each; however, the physiological variation did not outweigh the highly significant linear relationship between the daily cell division rate and the MC and NP net production rates. Surprisingly, the MC and NP contents per cell showed a maximum under P-PO_4-reduced and irradiance-reduced conditions. Both intra- and extracellular MC and NP concentrations were negatively related to P-PO_4 and irradiance. It is concluded that the proximate factor behind maximal cellular MC and NP contents is physiological stress.

Key index words: **dissolved toxins; eutrophication; *Microcystis*; *Planktothrix*; soluble reactive phosphorus**

Abbreviations: **DAPI, 4′,6-diamidino-2-phenylindol; HPLC-DAD, high performance liquid chromatography coupled to diode array detection; LC-MS, liquid chromatography coupled to mass spectrometry; MC, microcystin; NP, nostophycin**

Cyanobacteria are well known for their production of a multitude of highly toxic and/or allelopathic compounds. The toxic compounds include various cyclic peptides (the hepatotoxic MCs) and alkaloids (the potent neurotoxins and the hepatotoxic cylindrospermopsin), which have been studied both from a toxicological and a biological perspective (Chorus and Bartram 1999, Hudnell 2008). Several hypotheses have been formulated to explain the physiological regulation and the biological function of these toxic molecules, particularly of the MCs (Dittmann et al. 2001, Kaebernick and Neilan 2001, Kehr et al. 2006, Schatz et al. 2007, and others). For example, most frequently cited hypotheses include functions related to allelopathy, such as feeding deterrence and inhibition of other (eukaryotic) algae, and functions related to primary metabolism, such as iron scavenging, regulation of photosynthesis, and quorum-sensing like cell-cell communication processes (see Kaebernick and Neilan 2001, Schatz et al. 2007, Leao et al. 2009 for more recent reviews). Typically, planktonic cyanobacteria (*Anabaena*, *Microcystis*, *Planktothrix*) have been investigated for the regulation of toxin production under various environmental conditions (Sivonen and Jones 1999). Chemical structures of MCs produced by benthic cyanobacteria (e.g., *Nostoc* sp.) have been reported already during the 1990s (Sivonen et al. 1992, Beattie et al. 1998). The genus *Nostoc* is common in both terrestrial and aquatic habitats, typically growing on sediments or stones in the littoral or in running water (Komárek and Anagnostidis 1989, Dodds et al. 1995). Only recently, increasing evidence on the worldwide abundance of *Nostoc* sp. as an MC-producing organism has been reported (Oksanen et al. 2004, Mohamed et al. 2006, Wood et al. 2008, Bajpai et al. 2009, Oudra et al. 2009, Genuario et al. 2010). Indeed, it has been suggested that cyanobacteria growing on the sediments in reservoirs constitute a significant source of MC (Izaguirre et al. 2007) and should be included in routine monitoring for the presence of MC in raw water used for drinking water purification (Hurtado et al. 2008) or irrigation (Mohamed et al. 2006). In addition to MCs, a number of other bioactive compounds have been described in *Nostoc* sp. Prominently, the depsipeptides cryptophycins, which show strong cytotoxic effects as tubulin polymerization inhibitors, have been discovered during screening tests for bioactive activity (Golakoti et al. 1995). Other *Nostoc* sp. strains have been studied for their production of allelopathic compounds. For example, nostocyclamide,

[1]Received 7 October 2009. Accepted 23 August 2010.
[2]Author for correspondence: e-mail rainer.kurmayer@oeaw.ac.at.

a cyclic hexapeptide produced by *Nostoc,* inhibits growth in algae and bacteria (Jüttner et al. 2001). Becher et al. (2009) described nostocarboline that—functionally similar to anatoxin a(s)—is an inhibitor of acetylcholinesterase and the first serine protease inhibitor of an alkaloid structure that has been described. Hirata et al. (1996) described nostocine A, a violet pigment that occurred in the medium and inhibited the growth of various algae and cultured plants. Similarly, muscoride A, an oxazol alkaloid peptide, was reported to show weak antibacterial activity (Nagatsu et al. 1995). Gromov et al. (1991) described cyanobacterin from *Nostoc linckia,* which is nontoxic to mice, however, effective against *Synechococcus* at a concentration of 1 $mg \cdot L^{-1}$.

The strain *Nostoc* sp. 152 produces several MC structural variants (Namikoshi et al. 1990, Sivonen et al. 1992) and at least one other cyclic peptide, NP (Fujii et al. 1999). The MCs are cyclic heptapeptides that are defined by the presence of the ß-amino-acid residue (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) that is characteristic to the MC family (Diehnelt et al. 2006). While the MCs produced by *Nostoc* sp. 152 all show a structural modification in the Adda side chain, resulting in acetylated and demethylated MC structural variants (Sivonen et al. 1992), they all have been shown to retain their toxicity when compared to MC-LR [where LR refers to leucine (L) and arginine (A) in the positions 2 and 4 of the MC molecule, Sivonen et al. 1990]. Similarly to MC, NP is a cyclic hexapeptide that also contains a ß-amino-acid residue (2S,3R,5R)-3-amino-2,5-dihydroxy-8-phenyloctanoic acid (Ahoa), and so far, NP has been described to occur only in this strain. Since the MCs and NP have a ß-amino acid and the occurrence of two D-amino acids in common, it has been suggested that their synthesis is related (Fujii et al. 1999). However, in contrast to the toxin MC, NP showed no bioactivity (20 $\mu g \cdot mL^{-1}$) against several microorganisms (*Aspergillus, Candida, Staphylococcus, Bacillus,* and *Escherichia*) but weakly cytotoxic activity against lymphocytic mouse leukemia (Fujii et al. 1999). In this study, the physiological regulation of both MC and the related NP was studied under a regime of contrasting temperature, irradiance, and nutrient conditions. It is shown that both MC and NP were constitutively produced, but the cellular content was significantly increased under physiological stress conditions. The extent of the modulation, however, was not high enough to prevent a linear correlation between both MC and NP production rate and the growth rate.

MATERIALS AND METHODS

Growth experiments. All experiments were performed with the axenic strain *Nostoc* strain PCC9237 (*Nostoc* sp. strain 152 originally isolated from Lake Sääksjärvi, Finland, in 1986, Sivonen et al. 1990). *Nostoc* strain 152 was grown in O_2 medium (144 µM $P\text{-}PO_4$, 5.88 mM $N\text{-}NO_3$, Van Liere and Mur 1978). A 2^k levels factorial design was used, where k is the number of four factors that have been tested: temperature reduction (20°C vs. 12°C), irradiance reduction (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ vs. 1 µmol), $P\text{-}PO_4$ reduction (144 µM $P\text{-}PO_4$ vs. 0.14 µM), and $N\text{-}NO_3$-free conditions (5.88 mM $N\text{-}NO_3$ vs. $N\text{-}NO_3$ free). Cultures were illuminated from below and shaken once every day. The light intensity was determined using a quantum sensor (T. and J. Crump, Scientific instruments, Rayleigh, NC, USA) outside the culture flasks. Glassware used for the phosphate-reduction treatment was washed with sulfuric acid (10%, v/v) and deionized water to eliminate any possible external phosphate contamination. The design had 16 possible combinations that were tested in triplicate subsequently during 1 year.

In order to adapt the cells to the experimental conditions, precultures were established for a minimal duration of 3 months, and the growth was monitored by measuring absorbance at 880 nm (5 cm irradiance path) measured in a spectrophotometer (Shimadzu, Korneuburg, Austria, UV mini-1240). At the start of each growth experiment, precultures were diluted down to optical density (OD) 0.01 [23,000 ± 2,400 (1 SE) cells $\cdot mL^{-1}$] in a total volume of 800 mL, and aliquots of 100 mL were filled in eight 250 mL Erlenmeyer flasks to achieve optimal growth. Batch cultures were monitored every other day with the exception of extremely slow-growing cultures under $P\text{-}PO_4$ reduction and/or at 12°C (Fig. 1). A relatively wide variation in OD among treatments at 20°C, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (0.001–0.05) and at 12°C, 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (0.003–0.02) was observed at day 1 subsequent to the inoculation at day 0 (after 2 d). It is speculated that cell lysis subsequent to the inoculation can explain the extremely low OD readings at day 1 in some treatments. Cells were harvested at OD 0.10–0.15 at two consecutive sampling dates. Depending on the average growth rate, the two sampling dates were 2–3 d (at 20°C) or 2–6 d (12°C) apart. From these dates, cellular growth rates (d^{-1}) and MC and NP production rates (d^{-1}) were calculated using the formula

$$r = (\ln N_2 - \ln N_1)/\Delta t \quad (1)$$

where $N_{1,2}$ were the cell concentrations or peptide concentrations on consecutive sampling days (t_0, t_1) and Δt was the interval in days. Cells were fixed in 2% formaldehyde and counted using 4′,6-diamidino-2-phenylindol (DAPI) staining under an epifluorescence microscope (Carl Zeiss GmbH, Vienna, Austria, Axioplan) according to standard techniques (Porter and Feig 1980). Samples were filtered onto preweighed glass fiber filters (BMC Ederol, Vienna, Austria), dried at room temperature in a vacuum centrifuge (Eppendorf, Vienna, Austria, concentrator 5301), reweighed, and stored frozen at −20°C. Extracellular peptides were collected from the filtrate using solid phase extraction (SPE) via tC_{18} cartridges (Sep-Pak Vac 1cc, 100 mg, Waters, Dublin, Ireland) according to manufacturer's instructions. Cartridges were stored at −20°C. Peptides were eluted in 1 mL 90% MeOH, and extracellular extracts were stored at −20°C. Pilot experiments dissolving NP in O_2 medium and recollecting NP using SPE showed a recovery rate of 103 ± 2.4 (SE) %. The effective recovery of MC-LR (85.6 ± 12.6%) by using the same technique was shown during a previous study (Kosol et al. 2009).

To extract intracellular MC and NP, cells on filters (2 mg of dry weight) were extracted in 50% MeOH (v/v) after ultrasonification (Bandelin Electronic, Berlin, Germany) for 10 min. Extracts were shaken for 30 min and centrifuged at 16,000*g* (Thermo Scientific Heraeus, Schwerte, Germany, Biofuge Pico), and the clear supernatant was evaporated to dryness in a vacuum centrifuge at room temperature. This procedure was repeated three times, and the extracts were finally combined. Pilot experiments showed 99% extraction efficiency after three

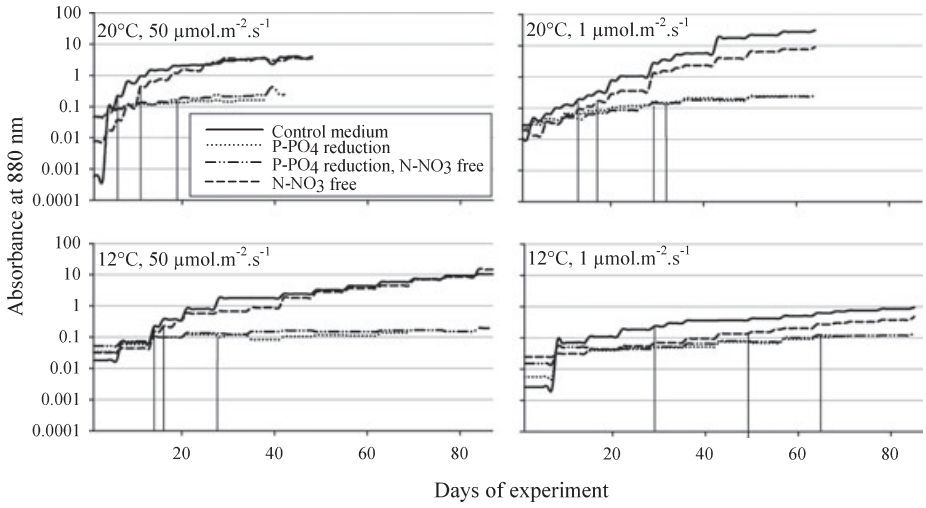

FIG. 1. Growth of axenic *Nostoc* strain 152 under high-irradiance (50 µmol · m^{-2} · s^{-1}) and low-irradiance (1 µmol · m^{-2} · s^{-1}) conditions, two different temperatures (12°C, 20°C), and P-PO_4-reduced and N-NO_3-free conditions. Growth has been monitored from day 1 by reading absorbance at 880 nm (mean value of three parallels) subsequent to inoculation. Vertical lines drawn from the growth curve down to the *x*-axis indicate the days of first harvest (t_0).

repetitions and optimal yields using sonification at room temperature compared without sonfication and/or extraction on ice.

For HPLC analysis, the samples were redissolved in 600 µL MeOH (50%, v/v) and centrifuged at 16,000*g*, and the cleared supernatant injected into HPLC-DAD. MCs and NP were detected at 210 nm using a linear gradient from 20% (v/v) acetonitrile [0.05% (v/v) trifluoroacetic acid] to 50% on a LiChrospher® 100, RP-18e (5 µm), LiChroCART® 250–4 cartridge system (Agilent, Vienna, Austria). NP (M+H 889, Fujii et al. 1999) eluted at 39 min, and MCs eluted at 40–44 min (M+H 1009, 1023, 1037, Namikoshi et al. 1990). NP was linearly detected from an area of 10,000 mAU (milli absorbance units) down to 100 mAU. Quantification was achieved using a calibrated standard provided by the University of Dundee (Louise Morrison, Geoffrey Codd). The calibration curve was $y = 1.6054x$ ($n = 8$, $R^2 = 1.0$), where y is the area recorded in mAU at 210 nm, and x is the amount of NP injected (in ng). The concentrations of MCs were determined as concentration equivalents of [Mdha, MeAsp]-MC-LR (Calbiochem [Part of Merck], VWR, Vienna, Austria). The linear regression line was $y = 1.471x$ ($n = 8$, $R^2 = 0.999$), where y is the peak area at 210 nm, and x is the injected amount of MC-LR standard (ng).

Multiple linear regression analysis was used to test the relationship between the days until harvest at OD 0.1, the number of cells/heterocysts per filament, intra- and extracellular MC and NP contents per cell, and the influence of temperature, irradiance, P-PO_4, and N-NO_3 concentrations as independent variables. A forward stepwise analysis was employed selecting for the independent variable for inclusion that makes the most significant unique (or additional) contribution to the prediction of the data. Calculations were performed using SPSS 15.0 for Windows (SPSS GmbH Software, Munich, Germany), and the *F*-value to enter the respective model was set default ($P < 0.05$). The data were log transformed in order to fulfill the assumptions of normality, constant variance, and multicollinearity.

RESULTS

Growth characteristics under the experimental conditions. In all 16 treatments, *Nostoc* strain 152 showed a significant increase in cell numbers from the day of inoculation ($OD_{880} = 0.01$) until the day of first harvest ($OD_{880} = 0.1$, min.-mean-max., 5-29-76 fold increase in cell numbers, Fig. 1). The number of days to reach $OD_{880} = 0.1$, however, differed significantly between treatments, and they were at maximum 10.9-fold higher under P-PO_4-reduced conditions (1 µmol · m^{-2} · s^{-1}, 12°C), that is, 70 ± 3 (1 SE) d when compared with optimal conditions (20°C, 50 µmol · m^{-2} · s^{-1}) in the full O_2 medium (6 ± 0 d) (Table 1). The cellular growth rate (ind.$^{-1}$ · d^{-1}) as calculated from cell numbers counted at the first (t_0) and the consecutive day (t_1) of cell harvest differed widely, that is, 0.003 ± 0.03 under P-PO_4-reduced and N-NO_3-free conditions (12°C, 50 µmol · m^{-2} · s^{-1}) versus 0.49 ± 0.11 under optimal conditions in the full medium (Table 1). Using multiple regression analysis, all four variables (i.e., the reduction in temperature, irradiance, P-PO_4, and N-NO_3) were included in the forward stepwise method and in total explained 89% of the variation that was observed among the days until first harvest (t_0, Table 2). As expected, temperature and irradiance reduction had the strongest influence on the reduction in cellular growth rate, while P-PO_4 reduction and N-NO_3 still contributed significantly.

Nostoc strain 152 formed long filaments under optimal or near-optimal growth conditions while filaments broke apart under P-PO_4-reduced conditions. On average, the number of cells per filament was 31 ± 0.9 (min. 6, max. 50). The number of cells per filament was more variable under P-PO_4-reduced conditions; at maximum it was reduced to 14.9 ± 5.6 at 50 µmol · m^{-2} · s^{-1} (12°C). The multiple regression approach revealed P-PO_4 and irradiance reduction as significant predictor variables; however, the explained variability was low (Table 2).

TABLE 1. Time until day of first harvest (t_0), growth rates and cell numbers (mean ± 1 SE) of axenic strain *Nostoc* strain 152 grown under high-irradiance (50 μmol · m^{-2} · s^{-1}) and low-irradiance (1 μmol · m^{-2} · s^{-1}) conditions, two different temperatures (12°C, 20°C), and phosphorus-reduced and nitrate-free conditions. t_0, t_1 = consecutive sampling days [2–3 d (20°C) or 2–6 d (12°C) apart].

	Time until day of first harvest (d)	Cells/fil	Heterocysts/fil	Vegetative cells/ heterocysts	Growth rate · d^{-1}	10^6 cells · mL^{-1}, t_0	10^6 cells · mL^{-1}, t_1
20°C, 50 μmol · m^{-2} · s^{-1}							
Full medium	6 ± 0	33 ± 0.4	1.2 ± 0.2	29 ± 2	0.493 ± 0.11	1.296 ± 0.125	3.535 ± 0.584
P-PO_4 reduced	15 ± 1	24 ± 4.7	0.2 ± 0.1	277 ± 209	0.049 ± 0.03	0.436 ± 0.042	0.487 ± 0.069
P-PO_4 reduced, N-NO_3 free	25 ± 4	29.6 ± 2.1	0.7 ± 0.2	133 ± 93	0.041 ± 0.04	0.527 ± 0.43	0.543 ± 0.096
N-NO_3 free	8 ± 1	30.6 ± 1.3	1.6 ± 0.2	19 ± 1	0.374 ± 0.08	1.539 ± 0.503	3.008 ± 0.642
20°C, 1 μmol · m^{-2} · s^{-1}							
Full medium	13 ± 0	34.8 ± 0.4	0.8 ± 0.1	46 ± 3	0.170 ± 0.04	0.658 ± 0.095	1.003 ± 0.217
P-PO_4 reduced	26 ± 2	36.8 ± 2.4	0.2 ± 0	302 ± 60	0.130 ± 0.02	0.486 ± 0.155	0.616 ± 0.17
P-PO_4 reduced, N-NO_3 free	27 ± 2	35.2 ± 1.7	0.7 ± 0.1	52 ± 3	0.027 ± 0.08	0.566 ± 0.032	0.636 ± 0.134
N-NO_3 free	19 ± 3	35.3 ± 1.4	1.8 ± 0.2	20 ± 1	0.177 ± 0.02	0.704 ± 0.105	1.128 ± 0.067
12°C, 50 μmol · m^{-2} · s^{-1}							
Full medium	15 ± 1	35.7 ± 0.1	1.2 ± 0.1	31 ± 1	0.286 ± 0.05	1.030 ± 0.273	2.018 ± 0.314
P-PO_4 reduced	36 ± 3	14.9 ± 5.6	0.1 ± 0	245 ± 107	0.053 ± 0.01	0.185 ± 0.015	0.219 ± 0.011
P-PO_4 reduced, N-NO_3 free	36 ± 3	34.9 ± 1.1	0.9 ± 0.1	42 ± 2	0.003 ± 0.03	0.354 ± 0.097	0.360 ± 0.098
N-NO_3 free	18 ± 3	35.7 ± 0.6	1.9 ± 0	19 ± 0.4	0.172 ± 0.01	1.256 ± 0.108	2.160 ± 0.366
12°C, 1 μmol · m^{-2} · s^{-1}							
Full medium	31 ± 2	33.5 ± 2.8	0.5 ± 0	74 ± 10	–0.032[1] ± 0.05	0.144 ± 0.028	0.132 ± 0.005
P-PO_4 reduced	70 ± 3	28.5 ± 0.9	0.3 ± 0	111 ± 14	0.037 ± 0.05	0.215 ± 0.055	0.249 ± 0.041
P-PO_4 reduced, N-NO_3 free	68 ± 2	32.3 ± 0.1	0.6 ± 0.1	62 ± 8	0.062 ± 0.03	0.238 ± 0.029	0.300 ± 0.01
N-NO_3 free	56 ± 4	26.2 ± 2.3	1 ± 0	26 ± 1	0.054 ± 0.04	0.515 ± 0.013	0.686 ± 0.119

[a]Cell growth was negative in two of three parallels as measured during the $t_0 - t_1$ interval (3 d).
[b]fil = filament.

TABLE 2. Parameters of multiple linear regression analysis used to test the influence of temperature (T), irradiance (I), P-PO_4 and N-NO_3 concentrations as independent variables (Var) on the days until harvest (at OD_{880nm} = 0.1), number of cells per filament, number of heterocysts per filament, intra- and extracellular microcystin (MC) and nostophycin (NP) contents per cell. The regression was $\log y = B_0 + B_1 \times \log X_1 + B_2 \times \log X_2 + B_3 \times \log X_3 + B_4 \times \log X_4$, where $X_{1–4}$ is the corresponding ($\log_{10}$ transformed) real value to be multiplied by the regression coefficients B_1–B_4.

	Var1	Var2	Var3	Var4	R^2 Var1	R^2 Var2	R^2 Var3	R^2 Var4	B_0	B_1	B_2	B_3	B_4
Days until day of first harvest	*T*	*I*	P-PO_4	N-NO_3	0.39	0.64	0.87	0.89	2.291	–0.037	–0.006	–0.002	–0.016
Cells · fil^{-1}	P-PO_4	*I*			0.05	0.098			1.468	0.001	–0.002		
Heterocysts · fil^{-1}	P-PO_4	N-NO_3			0.38	0.61			–0.304	0.004	–0.077		
Microcystin													
MC fg · $cell^{-1}$	*I*	P-PO_4			0.38	0.48			2.156	–0.006	–0.001		
Total MC fg · $cell^{-1}$	*I*	P-PO_4	*T*		0.28	0.46	0.51		2.404	–0.005	–0.001	–0.011	
Diss MC fg · $cell^{-1}$	P-PO_4	*T*			0.24	0.47			2.001	–0.003	–0.039		
% Diss MC	*T*	P-PO_4	*I*		0.27	0.38	0.44		1.621	–0.028	–0.001	0.003	
Nostophycin													
NP fg · $cell^{-1}$	P-PO_4	*I*	*T*		0.39	0.69	0.72		2.057	–0.003	–0.008	0.012	
Total NP fg · $cell^{-1}$	P-PO_4	*I*	*T*		0.55	0.75	0.77		2.24	–0.004	–0.007	0.011	
Diss NP fg · $cell^{-1}$	P-PO_4	*T*	*I*		0.67	0.73	0.78		1.56	–0.006	0.025	–0.005	
% Diss NP	P-PO_4	*I*	*T*		0.33	0.39	0.43		1.346	–0.002	0.003	0.009	

Diss = dissolved; fil = filament.

On average, one filament contained 0.9 ± 0.1 heterocysts (min. 0, max. 3). The formation of heterocysts depended on P-PO_4 and N-NO_3 reduction only (R^2 = 0.61). When compared to the growth conditions in the full medium at 50 μmol · m^{-2} · s^{-1} (20°C), the number of heterocysts per filament was increased by 0.8- to 1.6-fold under conditions of N-NO_3 reduction. However, it was decreased to 0.07- to 0.23-fold under conditions of P-PO_4 reduction. From the considerable variation in the growth rate as well as the ratio of vegetative cells/heterocysts that was found, it is concluded that environmental conditions both limiting and nonlimiting to growth were observed under the experimental design.

Cellular content of MC and NP under the experimental conditions. In general, the net production of MC and NP continued under all experimental conditions. Taking all data together, no difference between the average total content including both intra- and extracellular MC (119 ± 7 fg · $cell^{-1}$) and NP (120 ± 8 fg · $cell^{-1}$) was detected (paired *t*-test, P = 0.87, df = 94). In contrast, the percentage of the average amount of extracellular MC (19 ± 1.5%) was lower when compared with the average amount

of extracellular NP (29 ± 1.5%), (paired *t*-test, $P < 0.001$, df = 94). Vice versa, the average intracellular MC content (98 ± 5 fg · cell^{-1}) was significantly higher than the average intracellular NP content (83 ± 5 fg · cell^{-1}) (paired *t*-test, $P < 0.001$, df = 95, Table S2 in the supplementary material).

When compared with optimal growth at 50 μmol · m^{-2} · s^{-1} (20°C) in the full O_2 medium, the total (intra- and extracellular) MC and NP contents varied at maximum 5-fold and 10-fold, each. According to multiple regression analysis, the variables P-PO_4, irradiance, and temperature reduction had the strongest influence on the variation as observed among the intra-/extracellular MC and NP contents (Table 2). In contrast to its effect on growth, the factor N-NO_3 reduction was never included as a predictor variable by the multiple regression analysis. The majority of the regression coefficients were negative, implying significant negative relationships between predictor variables and the respective dependent variable (see Table S1 in the supplementary material for Pearson product-moment correlation coefficients between $\log_{10}$-transformed variables).

The total MC cellular contents were 2.2- to 5.0-fold increased under irradiance-reduced conditions, while P-PO_4 reduction led to 2.2- to 3.6-fold increase in the cellular MC content (Fig. 2). Accordingly for both intra- and extracellular MC contents, the factors irradiance, P-PO_4, and temperature reduction were found to best predict the variation that was recorded. Irradiance, P-PO_4 reduction, and temperature were all negatively related to MC. The reduction in P-PO_4 also had the most significant effect on total NP net production. When compared with optimal conditions (50 μmol · m^{-2} · s^{-1}, 20°C, full O_2 medium), the total NP cellular contents were 4.4- to 10.6-fold higher under conditions of P-PO_4 reduction at any irradiance and temperature condition or their combination. For the total NP cellular content, and both intra- and extracellular NP contents, P-PO_4 reduction and, to a lesser extent, irradiance and temperature reduction were identified as predictor variables explaining the variation that was observed (Table S2, Fig. 2). Both P-PO_4 reduction and irradiance were significantly negatively related to NP, while temperature showed a significant positive relationship. In summary, P-PO_4 reduction and reduced irradiance led to a pronounced increase in the cellular MC and NP contents.

NP and MC net production rates. Because of the large physiological variation that was determined, it was of interest to find out whether cell numbers could still be used as a variable to predict MC and NP net production in water. Taking the data from all experiments together, peptide net production rates were plotted against the cell production rates and analyzed using linear regression analysis. The highest MC and NP production rates were observed in the most rapidly growing cultures. For both

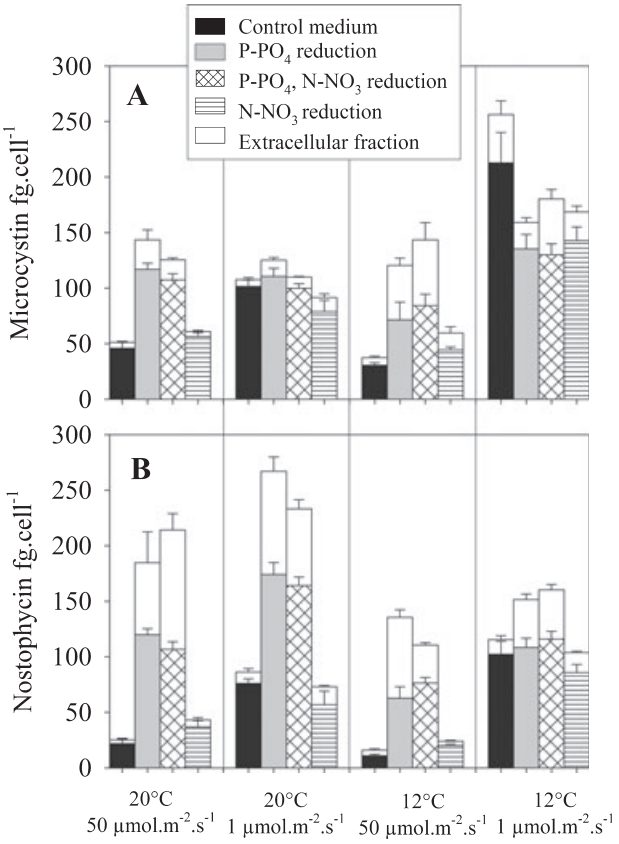

FIG. 2. Mean (+1 SE) concentration of intra- and extracellular (A) microcystin (MC) and (B) nostophycin (NP) contents (fg · cell^{-1}) of *Nostoc* strain 152 under four different environmental conditions and their combinations. High-irradiance (50 μmol · m^{-2} · s^{-1}) and low-irradiance (1 μmol · m^{-2} · s^{-1}) conditions, two different temperatures (12°C, 20°C), and P-PO_4-reduced and N-NO_3-free conditions. For each treatment combination, the extracellular fraction is shown on the top of each column (in white).

peptides, a significant linear relationship between the daily production of biovolume (cell growth) and the daily production of MC or NP was found (Fig. 3): MC, $y = -0.02 + 1.07x$ ($R^2 = 0.76$); NP, $y = 0.01 + 1.12x$ ($R^2 = 0.7$), where y and x were the $\log_{10}$-transformed peptide production rates (d^{-1}) and cellular growth rates (d^{-1}), respectively. Notably, the slope of both regression curves was close to 1, and the origin of the regression curves was close to 0. In summary, for both peptides, the physiologically induced variation among cellular peptide contents did not outweigh the overall influence of the daily cellular growth rate on peptide net production.

DISCUSSION

According to this study, the highest MC and NP contents per cell were observed under severe growth-limiting conditions, which were mostly due to P-PO_4 limitation. During the experiments, *Nostoc* strain 152

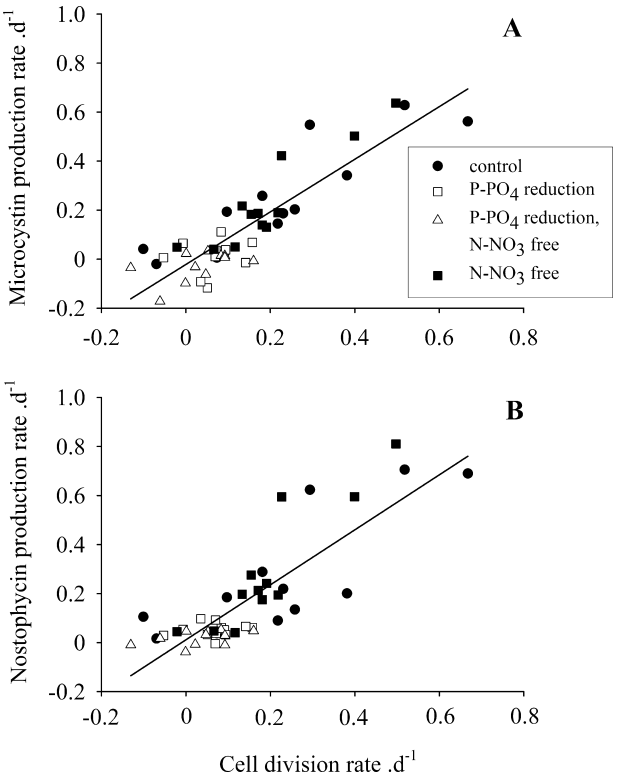

FIG. 3. Relationship between the cell division rate (d^{-1}) and (A) the net MC production rate and (B) the net NP production rate (d^{-1}) of *Nostoc* strain 152. The regression curves were fitted by a linear regression calculation and least-square approximation. The $P\text{-}PO_4$-reduced treatments are indicated by open symbols; the closed symbols indicate the full medium and $N\text{-}NO_3$-free medium. For details on regression curves, see text.

was grown under $P\text{-}PO_4$ and $N\text{-}NO_3$ reduction conditions at 12°C under both irradiance levels for >12 months (including the time of preculturing). Therefore, the cells were considered to be acclimatized to the experimental conditions. The concentration of 0.144 μM of $P\text{-}PO_4$ is corresponding to the lower threshold used to define the mesotrophic state of surface water (Vollenweider and Kerekes 1982). During the experiments, mostly linear growth of the cells under the various treatments has been observed (Fig. 1). This finding implies that the measured growth rates (Table 1) do not correspond to the exponential growth phase but rather indicate an average growth rate as observed under the various treatment conditions. Accordingly, a slow-growing culture can be expected to show an increase in MC and NP production if the maximum intrinsic growth rate would have been recorded, for example, as realized under continuous culture conditions. On the other hand, the maxima of the cellular growth rates as reported in this study correspond with the growth rates as determined for other planktonic MC-producing cyanobacteria under continuous culture conditions (Long et al. 2001, Wiedner et al. 2003, Tonk et al. 2005). The 5-fold variation on cellular MC contents (31–213 fg MC · $cell^{-1}$) observed during this study compares with the variations reported from continuous cultures (3-fold, Long et al. 2001; 2-fold, Wiedner et al. 2003; 5-fold, Tonk et al. 2005). For the planktonic genera *Anabaena*, *Microcystis,* and *Planktothrix,* rather similar cellular MC contents have been reported, that is, <1–2 mg of MC · g^{-1} of dry weight in *Anabaena* (Rapala et al. 1997), 0.02–0.53 mg of MC · g^{-1} dry weight of *Microcystis* sp. (Saker et al. 2005), 1.7 ± 0.3 (max. 4.5) mg of MC · g^{-1} dry weight, and 1.2 ± 0.2 (max. 4.5) mg of MC · g^{-1} dry weight for *Planktothrix agardhii* and *Planktothrix rubescens*, respectively (Kosol et al. 2009). In contrast, for *Nostoc* sp., MC contents have been reported that are lower when compared with planktonic cyanobacteria (0.139 mg of MC · g^{-1} of dry weight, Oudra et al. 2009; 0.025 mg of MC · g^{-1} of dry weight, Bajpai et al. 2009; 0.2 mg of MC · g^{-1} of dry weight, Oksanen et al. 2004). In this study, cellular MC and NP production levels compare with those reported for *Microcystis* and *Planktothrix* (Wiedner et al. 2003, Tonk et al. 2005). Recently, Kaasalainen et al. (2009) reported 0.4 mg of MC · g^{-1} of dry weight for *Nostoc* strain UK18 that has been isolated from the lichen *Peltigera leucophlebia*. Consequently, it is concluded that there is not a principal difference in MC content between planktonic and benthic MC-producing genera.

It is striking, however, that in contrast to *Microcystis* and *Planktothrix,* the cellular MC content in *Nostoc* strain 152 was negatively related to light availability. While for the genera *Microcystis* and *Planktothrix,* a 3- to 4-fold higher cellular MC content at 50 μmol · $m^{-2} \cdot s^{-1}$ when compared with 1 μmol · $m^{-2} \cdot s^{-1}$ has been reported (Wiedner et al. 2003, Tonk et al. 2005), the cellular contents of both MC and NP of *Nostoc* strain 152 were highest under conditions of 1 μmol · $m^{-2} \cdot s^{-1}$. It is speculated that this principal difference might be caused by the nonplanktonic origin of the strain 152. Although *Nostoc* strain 152 has been isolated from a water bloom (Sivonen et al. 1990), it is likely that it originated indeed from soil due to washout after precipitation for the following reasons: (i) When collected, the water bloom sample was dominated by *Aphanizomenon flos-aquae*. The mouse bioassay of the bloom sample indicated that it was nontoxic (50% lethal dose, intraperitoneally, mouse, >1,500 mg · kg^{-1}), implying that the abundance of *Nostoc* sp. strain 152 in the plankton community could not be high. (ii) The same acetylated and demethylated MC structural variants (ADMAdda variants, Sivonen et al. 1992) as observed in *Nostoc* strain 152 have been identified from a *Nostoc* strain originally isolated from lichens (Oksanen et al. 2004) and in *Nostoc* sp. occurring in cyanobacterial mats of Antarctica (Wood et al. 2008). With one exception (*P. agardhii*, Laub et al. 2002), the occurrence of the rare acetylated and demethylated Adda MC variants has only been reported from *Nostoc* sp. (Beattie et al. 1998,

Oksanen et al. 2004). Therefore, it is most likely that *Nostoc* strain 152 originated from a benthic habitat, as it is well known that *Nostoc* sp. occurs as epilithic algae on stones or as epipelic algae on sediments.

Notably, although the P-PO_4-reduced and irradiance-reduced conditions significantly increased the MC content per cell up to 5-fold and the NP content per cell up to 10-fold, the physiologically induced variation did not outweigh the general relationship between cell division and the MC and NP net production rates. In their seminal paper, Orr and Jones (1998) concluded that for individual strains of cyanobacteria, MC net production depends primarily on the cellular growth rate, while environmental conditions influence MC production rather indirectly via the cellular growth rate. Basically, the Orr and Jones hypothesis was built on the overall observation that—although MCs and other toxins, such as anatoxin a, are clearly secondary metabolites—environmental factors may affect their content in cyanobacteria, but only within a range of less than an order of magnitude (Sivonen and Jones 1999). As the hypothesis of a noninducible continuous production of cyanotoxins has never been disproved, the monitoring approach to use cyanobacterial biovolume as a proxy to estimate MC production in surface water has become more widely accepted (Bartram et al. 1999, Rogalus and Watzin 2008).

In summary, the results show that although the synthesis of MC and NP is clearly regulated in response to low P-PO_4 concentrations, and low-irradiance and temperature conditions, the net production rate of both compounds is related to the cell division process or population growth. This implies that the synthesis of both peptides is highly integrated into the primary metabolism. While this has been found for planktonic genera such as *Anabaena*, *Microcystis,* and *Planktothrix*, it has never been reported for benthic cyanobacteria. From *Nostoc,* a surprising type of physiological regulation of MC production in response to the environment has been observed. Caution is needed when the type of regulation as observed in one single strain/species is extrapolated to other strains or more distantly related MC-producing cyanobacteria.

I am grateful to Anika Stracke, Josef Knoblechner, Gertraud Roidmayr, and Eva Schober for their technical assistance in the laboratory, and Louise Morrison (University of Dundee) for preparing the NP standard. The *Nostoc* strain PCC9237 was provided by Nicole Tandeau de Marsac (Institute Pasteur, Paris). Thomas Rohrlack (NIVA, Oslo) identified the MCs by means of LC-MS. I am grateful to two anonymous reviewers for their critical comments. This study was supported through the EU project PEPCY (Contract No QLRT-2001-02634) and the Austrian Science Fund (P20231).

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Pearson product-moment correlation coefficients between $\log_{10}$-transformed variables [time until day of first harvest, cellular microcystin (MC) and nostophycin (NP) contents] and $\log_{10}$-transformed experimental factors (temperature, irradiance, P-PO_4, N-NO_3). Significant correlations (one-tailed significance $P < 0.05$) are underlined. Experimental factors that were included in the multiple regression models are indicated by gray shading. The B1 predictor variable as identified by the forward multiple regression procedure is printed in bold.

Table S2. Intracellular and extracellular microcystin (MC) and nostophycin (NP) contents in $fg \cdot cell^{-1}$ (mean ± 1 SE) of strain *Nostoc* strain 152 grown under high-irradiance ($50\ \mu mol \cdot m^{-2} \cdot s^{-1}$) and low-irradiance ($1\ \mu mol \cdot m^{-2} \cdot s^{-1}$) conditions, two different temperatures (12°C, 20°C), and P-PO_4-reduced and N-NO_3-free conditions.

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