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Variation in peptide net production and growth among strains of the toxic cyanobacterium *Planktothrix* spp.

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Cyanobacteria frequently form mass developments in surface waters. Populations consist of strains that differ in the production of bioactive peptides, e.g. microcystins (MC) inhibiting protein phosphatases 1 and 2A and anabaenopeptins (APN) inhibiting carboxypeptidases. Forty-nine strains (18 green-pigmented (phycocyanin-rich) *P. agardhii* strains and 31 red-pigmented (phycoerythrin-rich) *P. rubescens* strains) of the filamentous cyanobacterium *Planktothrix* (*Anagnostidis et Komarék*) were analysed for their MC and APN net production rates. These rates were compared with (i) the pigmentation, (ii) the proportion of extra- and intracellular peptide concentrations and (iii) the cellular growth rates under standardized laboratory conditions. Excluding the strains lacking MC and APN, the MC and APN contents varied up to 14-fold and 12-fold, each. The variation in minimum and maximum peptide content ($0.32\text{--}4.51\ \mu\text{g MC mg}^{-1}$ dry weight; $0.85\text{--}10.32\ \mu\text{g APN mg}^{-1}$ dry weight) exceeded the variation found for chlorophyll *a* ($4.8\text{--}16.9\ \mu\text{g mg}^{-1}$ dry weight). The extracellular proportions of MC (0–62%) and APN (0–58%) varied among strains, however, on average, proportions of extracellular MC and APN were low (MC: $8.8 \pm (1\ \text{SE})\ 1.9\%$, APN: $8.4 \pm 1.8\%$). Among all strains cellular growth rates showed a 5-fold variation ($0.07\text{--}0.33$ doublings of dry weight $\cdot\text{day}^{-1}$) and were found independent of the pigmentation and the peptide net production rate. It is concluded that the MC and APN net production rate is not causally related to the cell-division cycle and the synthesis of highest amounts of MC and APN does not constrain cell division.

Key words: cyanobacteria, cyanotoxins, freshwater phytoplankton, harmful algal bloom, growth rate, high performance liquid chromatography, *Microcystis*, water reservoir

Introduction

Because of their frequent occurrence in fresh water, toxin-producing cyanobacteria are considered to be a general threat to drinking water quality (Codd *et al.*, 1999; WHO, 2004). Many of the studies dealing with toxin production have focused on microcystins (MC), cyclic heptapeptides inhibiting eukaryotic proteinphosphatase 1 and 2A. MCs are synthesized via non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) assembled into large multifunctional proteins encoded by the *mcy* gene cluster (Tillett *et al.*, 2000). The chemical structure of MC is cyclo (D-Ala¹-X²-D-MeAsp³-Z⁴-Adda⁵-D-Glu⁶-Mdha⁷), where D-MeAsp is the non-proteinogenic amino acid D-erythro- β -iso-aspartic acid (methyl aspartate), Mdha is N-methyl-dehydroalanine and Adda is a β -amino acid with a C₁₀-chain: (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. X and Z represent variable L-amino acids in positions 2 and 4, respectively (Carmichael, 1992). Similar to MCs,

anabaenopeptins (APN) have been reported to inhibit protein phosphatase 1 (APN A and B; Gkelis *et al.*, 2006) as well as proteases such as carboxypeptidase A (APN G and H, Itou *et al.*, 1999). APNs contain six amino acids: five of them form a cyclic structure with a sixth amino acid that is attached. The pentapeptide moiety has the common structure cyclo (D-Lys⁵-L-Val⁴-L-Hty³-L-MeAla²-L-Phe¹) with L-Arg⁶ (or L-Tyr) bound to D-Lys⁵ through an ureido linkage. L-Hty is L-homotyrosine and MeAla is N-methyl-alanine (Harada *et al.*, 1995; Fujii *et al.*, 2002). D-Lys⁵ is the only non-variable amino acid in APN. APNs are synthesized by NRPS analogously to the MCs (Fujii *et al.*, 2002).

Regulation of MC net production in single isolates (strains) has been addressed by a number of studies quantifying the effects of various environmental conditions on MC content in the laboratory, e.g. effects of temperature, pH, irradiance, macronutrients (N, P), and trace elements (Sivonen & Jones, 1999). Contrary to expectations the production of MCs was not inducible and different environmental factors were found to induce moderate changes in MC content only.

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These results are in contrast to the regulation of antibiotics and sporulation factors via NRPS, which is induced or increased as growth declines (Marahiel *et al.*, 1993). For example during the transition from the logarithmic to the stationary phase of growth in batch culture, *Bacillus subtilis* shows increased production of the peptide antibiotics tyrocidine and gramicidine S and the lipopeptide surfactin. Orr & Jones (1998) suggested that MC production is coupled primarily to cell division of the organism and concluded that MC – although it is clearly a secondary metabolite – rather displays the attributes of essential intracellular nitrogenous compounds. According to this theory the major influence of all possible environmental factors is indirect – through their effect on cell division and growth, whereas the direct effects on MC biosynthesis are of minor importance. The specific growth rate has been used to predict the intracellular MC content under nitrogen-limiting conditions in culture (Long *et al.*, 2001). The model of Long *et al.* (2001) implies that MC production is constitutive and MC-producing strains will always contain at least a minimal intracellular MC content and a maximum content determined by the nutrient saturated growth rate (for a given temperature and light growth conditions).

In contrast to the regulation of MC synthesis within a strain, very few studies have investigated the phenotypic variation in MC content between strains. In a few cases large differences in MC content related to the dry weight of the strains have been reported (Bolch *et al.*, 1997; Saker *et al.*, 2005). For *Planktothrix* 10-fold and 16-fold differences in MC content related to dry weight have been reported (Kurmayer *et al.*, 2005; Welker *et al.*, 2004). While dry weight is usually considered rather inaccurate to estimate the MC content it is completely unclear how the MC production rate relates to the differences in growth rates among strains as well as to the differences in the production of other peptides.

In this study 49 strains of *Planktothrix* were analysed for their MC and APN contents and their specific peptide net production rates during conditions of maximum specific growth (μ_m , sensu Kohl & Nicklisch, 1988). The genus *Planktothrix* (*Oscillatoria*) (Anagnostidis & Komarek, 1988) occurs frequently in lakes and reservoirs in the temperate zone of the Northern Hemisphere. The species *P. rubescens* and *P. agardhii* differ in pigmentation and planktonic life-form: *P. rubescens* is typically found in deep, stratified and oligo- to mesotrophic waters, while *P. agardhii* occurs in shallow and more eutrophic waters. Chromatic adaptation has not been observed within *Planktothrix* (Skulberg & Skulberg, 1985) and the hydrostatic pressure and the underwater

light climate act as factors leading to the ecological divergence between both life forms (Beard *et al.*, 2000; Davis *et al.*, 2003). In some ecosystems both species have been reported to co-occur for years, for example in Blelham Tarn, United Kingdom (Davis *et al.*, 2003) or in Lake Steinsfjorden, Norway (Halstvedt *et al.*, 2007).

Material and methods

Origin and strain cultivation

Forty-nine strains (18 *P. agardhii*, 31 *P. rubescens*) were isolated from different lakes as described previously (Kurmayer *et al.*, 2004, see supplementary material available at <http://www.informaworld.com/mpp/uploads/downloadfile.pdf>) and assigned to *P. agardhii* (green-pigmented) or *P. rubescens* (red-pigmented) according to Suda *et al.* (2002). All strains were grown in BG₁₁ medium under sterile conditions (Rippka, 1988) at 20°C and 40–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Philips LTD, 36W/965, Vienna, Austria). A few strains have been purified axenic prior to the growth experiments (No. 70/1a, No. 160/1a, No. 12/1a, PCC 7821, No. 14/1a, No. 90/1i, No. 113/1b). In order to adapt the cells to the experimental conditions pre-cultures for a minimal duration of 3 months were established and their growth was monitored by measuring optical density (OD) at 880 nm (5-cm light path). At the start of each growth experiment pre-cultures were diluted down to $\text{OD}_{880} = 0.01$ ($3 \times 10^4 - 16 \times 10^4 \text{ cells ml}^{-1}$) in a total volume of 800 ml and aliquots of 100 ml were distributed over eight Erlenmeyer flasks (250 ml) to achieve optimal growth. Cultures were grown semi-continuously and growth was monitored by OD readings every other day. Because not all strains could be grown simultaneously one strain (*P. agardhii* strain No. 32) was included as a control strain to standardize for uncontrolled factors. Each strain was tested in duplicate at two consecutive sampling dates.

Cell harvesting

Cells were harvested at $\text{OD} = 0.1-0.15$ at two consecutive sampling dates, usually between days 4 and 19 depending on the strain-specific growth rate. Growth rates (r) (day^{-1}) and peptide net production rates (day^{-1}) were calculated using the formula, $r = (\ln N_2 - \ln N_1) / \Delta t$, where $N_{1,2}$ were the cell concentration/peptide concentrations at consecutive sampling days and Δt was the interval in days (3 days). Cells were fixed in 2% formaldehyde and filtered onto IsoporeTM membrane filters (0.2 μm GTBP Ø 25 mm, Millipore), stained with DAPI and enumerated using epifluorescence microscopy. A minimum of 400 specimens was counted. As individual cells in the filaments of *Planktothrix spp.* could not always be distinguished, the filament length was estimated using the boxes of the counting grid (at 400 \times magnification). For 20 randomly selected filaments the cell number per box of the counting grid was determined and the average cell number per box was used to calculate cell numbers. Analogously biovolume

was determined by measuring the diameter of 20 randomly selected filaments at 1125 \times magnification and calculating biovolume by assuming a cylindrical shape.

Samples were harvested by filtering onto pre-weighed glass fibre filters (BMC Ederol, Vienna), dried in a vacuum centrifuge, weighed and stored frozen at -20°C . A minimum of 1.0 mg of dry weight of biomass was collected. Dissolved peptides were collected from the filtrate using solid phase extraction (SPE) via tC_{18} cartridges [Waters, Sep-Pak Vac 1cc (100 mg)] according to standard techniques. Cartridges were stored at -20°C . After acclimation to room temperature the cartridges were washed with 10 ml H_2O and 10 ml 20% (v/v) methanol and peptides were eluted in 1 ml 90% MeOH and dissolved extracts were stored at -20°C until HPLC analysis. During pilot experiments the spiking of BG₁₁ medium (300 ml) with 1 μg MC-LR standard revealed an average recovery rate of $85.6 \pm 12.7\%$ (95% confidence limit, $n=6$) as measured by HPLC. For 1 μg of APN B the recovery rate was $99 \pm 0.4\%$.

Peptide extraction and HPLC analysis

To extract MC and APN filters were cut into pieces and extracted in 50% MeOH (v/v). Extracts were shaken for 30 min, centrifuged at 16,000 g and the clear supernatant was evaporated to dryness in a vacuum centrifuge. This procedure was repeated twice. Pilot experiments showed 99% extraction efficiency after three repetitions and optimal yields with extracting on ice and without sonification compared with extraction at room temperature with sonification (strain No. 40).

The extracts were analysed for MCs and APNs by high performance liquid chromatography (HPLC) coupled to diode array detection (HPLC-DAD). For HPLC analysis the samples were re-dissolved in 600 μl MeOH (50/50, v/v), centrifuged at 16,000 g (10 min) and the cleared supernatant was injected. MCs and APNs were quantified at 240 nm and 210 nm, each using a linear gradient from 20% acetonitrile (0.05% TFA) to 50% acetonitrile on a LiChrospher[®] 100, ODS, 5 μm , LiChroCART[®] 250-4 cartridge system (Agilent, Vienna, Austria). Structural variants of MCs and APNs were assigned using the information obtained from UV spectra and retention times and identified by sensitive matrix assisted desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) as described (Kurmayer *et al.*, 2004, 2005). In order to evaluate the reproducibility of the detection of MCs and APNs from aqueous-methanolic extracts via the HPLC-DAD technique the following pilot experiment was performed (Welker & Erhard, 2007): the biomass of ten strains (Nos 40, 67, 77, 82, 83, 95, 101, 110, 173, 208, CCAP 1460/5) was (i) extracted as described above and analysed by HPLC-DAD coupled to MALDI-TOF MS and (ii) directly analysed by single filament MALDI-TOF MS as described (Kurmayer *et al.*, 2004). This comparison revealed that all of the MCs and APNs present in a specific strain were detected using the linear gradient in HPLC-DAD as described above. Further, the retention time and the UV-spectra were useful to identify the

elution of APN B (16.3–17 min), APN F (18.9–19.5 min), APN A (20.7–21.3 min), oscillamide (OSC) Y (23.4–23.7 min) and those of MCs from ([Asp, Mdha]-MC-RR (25.8–26.2 min), [Asp, Dhb]-MC-RR (26.5–26.9 min), [Asp]-MC-HtyR (32.6–33.3 min), [Asp]-MC-LR (33.2–34 min).

In addition standards have been used to identify novel MCs (35–36.1 min), the structure of which has been elucidated as [Asp, Dhb]-MC-HtyY, m/z 1074 and [Asp, Dhb]-MC-HtyHty, m/z 1088 (R. Kurmayer & T. Hemscheidt, unpublished). Standards of the variants APN 908 (m/z 909) and APN 915 (m/z 919) were used to identify the more rare variants APN J (18.4–18.9 min) and APN I (22.8–23.2 min), respectively (H. Okumura, B. Philmus & T. Hemscheidt, unpublished). The concentrations of MCs were determined as concentration equivalents of [MeAsp, Mdha]-MC-LR (Calbiochem, Schwalbach, Germany). The linear regression was $y=1.9525x$ ($R^2=1$), where y was the peak area at 240 nm and x was the injected concentration of microcystin-LR standard (μg). The concentrations of APNs were determined as concentration equivalents of APN B using a purified standard (Judith Blom, University of Zürich, Institute of Plant Biology, Limnological Station) and quantification via the molar extinction coefficient ($\lambda_{\text{max}}=225$ nm, $\epsilon=8833$, Harada *et al.*, 1995). The linear calibration regression was $y=1.3169x$ ($R^2=1$), where y was the area recorded at 210 nm and x was the concentration of APN injected (μg). Under the conditions as described above the detection limit was 10 ng both for the MC-LR and the APN B standard.

For chlorophyll *a* analysis, filters containing aliquots of the cells that were harvested for peptide analysis, were extracted in 1 ml of 90% acetone (v/v) and grinded with a mortar under dim light. Four ml of 90% acetone were added and the extraction was performed in the refrigerator over night. Two ml of the suspension were centrifuged (10 min, 16,000 g , 4°C) and 100 μl of the clear supernatant were injected into HPLC-DAD using a linear gradient of three solvents: (A) methanol (80:20 methanol: 0.5 M ammoniumacetate, v/v), (B) acetonitrile (90%) and (C) acetic acid ethyl ester, as developed by Wright *et al.* (1991). A Superspher[®] 100 RP-18, 4 μm , 250-4 cartridge system (Agilent, Vienna, Austria) was used. Concentrations in μg per mg dry weight were estimated for chlorophyll *a* with calibration curves using a standard supplied by DHI Water & Environment (Hørsholm, Denmark). For determination of the acid ratio A/A_a (the ratio of chlorophyll *a* to magnesium-free phaeophytine) samples were acidified with 0.1 M HCl according to standard protocols (Lorenzen, 1967). $A/A_a \sim 1.7$, if only undegraded chlorophyll *a* is present and $A/A_a \sim 1.0$, if only degradation products of chlorophyll *a* are present.

Results

Variation of peptide contents within and among strains

Three methods of biovolume estimation were compared in their ability to predict the

concentration of MC and APN in cells of the control strain No. 32: cellular dry weight (DW), cell counting and biovolume estimated from the microscope. All three methods gave highly significant correlations between peptide concentrations and the estimated biovolume (Fig. 1). The regressions were for dry weight: $y = 2193x + 28$ ($n = 35$, $R^2 = 0.8$, MC) and $y = 2718x + 57$ ($n = 35$, $R^2 = 0.83$, APN), for cell numbers: $y = 5 \times 10^{-5}x + 31.7$ ($n = 35$, $R^2 = 0.87$, MC) and $y = 6 \times 10^{-5}x + 72.7$ ($n = 35$, $R^2 = 0.82$, APN), for biovolume: $y = 2716x + 31$ ($n = 35$, $R^2 = 0.89$, MC) and $y = 3154x + 71$ ($n = 35$, $R^2 = 0.85$, APN), where y was the peptide concentration (ng ml^{-1}) and x was the biovolume concentration.

In total 35 strains produced MCs, while 46 strains contained APNs. The cellular concentration of both MC and APN was variable between strains, i.e. the MC and APN contents varied 14-fold and 12-fold, each (excluding the strains lacking MC or APN). In order to find out whether this variability was due to analytical errors or due to strain-specific differences the variability in peptide contents found within the control strain was compared with the variability found between strains (Fig. 2). The variation found between strains was significantly higher when compared with the variation found within the control strain (Mann–Whitney rank sum test, $p < 0.001$).

Peptide contents and peptide composition

In general all three biovolume estimates (dry weight, microscopical determination of cell numbers and microscopical determination of cyanobacterial biovolume) showed highly consistent results (Table 1). The counting of cells under the microscope did not improve the errors of the estimates, which was attributed to the unequal distribution of aggregate forming filaments on the filters as mentioned earlier (Repka *et al.*, 2004). Only the contents related to dry weight are reported in the following.

The contents of MC and APN were found in the same order of magnitude and were 15-fold (MC) or 19-fold (APN) lower (excluding the strains with zero peptide content) when compared with the chlorophyll *a* content (Table 1). The two species differed significantly in chlorophyll *a* content (*P. agardhii*: mean \pm SE, $13.1 \pm 2.4 \mu\text{g mg}^{-1}$ DW; *P. rubescens*: $8.0 \pm 1.8 \mu\text{g mg}^{-1}$ DW; *t*-test, $p < 0.001$). No significant difference (*t*-test, $p = 0.124$) in MC content between species was found: Among *P. agardhii* strains the MC content (mean \pm SE, $1.7 \pm 0.3 \mu\text{g mg}^{-1}$ DW) varied between zero (four strains) and $4.5 \pm 0.4 \mu\text{g mg}^{-1}$ DW (No. 31/1), while in *P. rubescens* the MC content (mean \pm SE, $1.2 \pm 0.2 \mu\text{g mg}^{-1}$ DW) was found

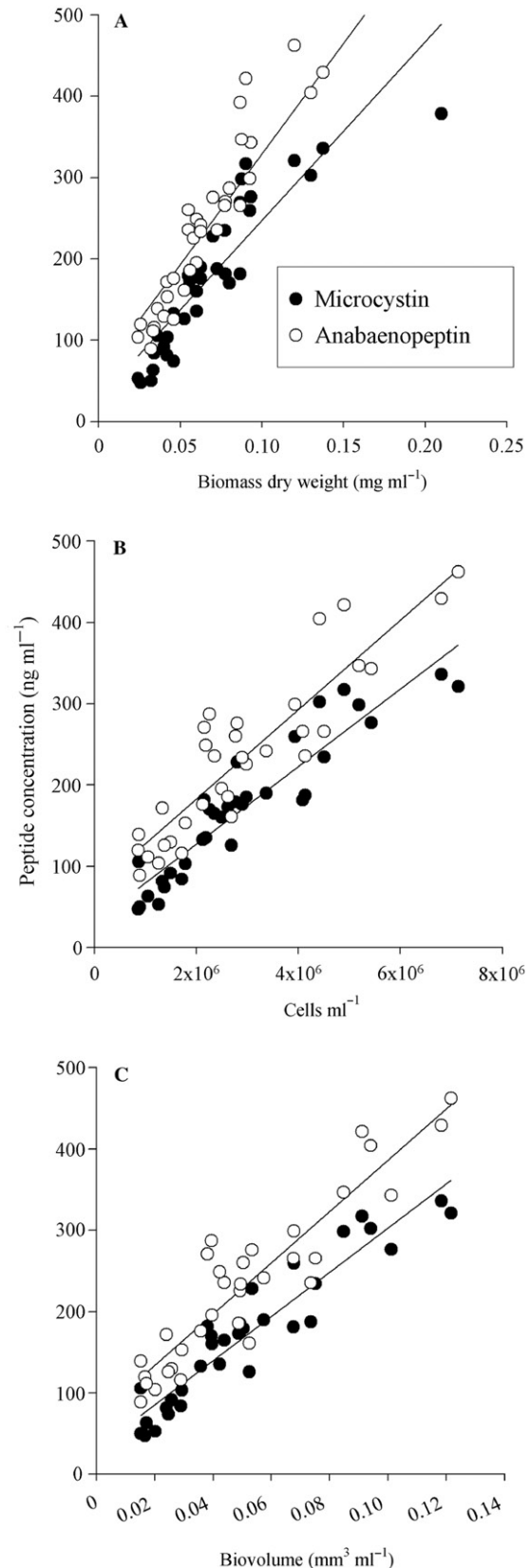


Fig. 1. Relationship between microcystin (MC; black circles) and anabaenopeptin (APN; white circles) concentrations and biovolume of the filamentous cyanobacterium *Planktothrix* strain No. 32. Biovolume was estimated by (A) dry weight (regression coefficients for MC: $R^2 = 0.8$ and APN $R^2 = 0.83$) (B) cell counting (MC: $R^2 = 0.87$ and APN $R^2 = 0.82$) and (C) calculating biovolume from filaments by assuming a cylindrical shape (MC: $R^2 = 0.89$ and APN $R^2 = 0.85$).

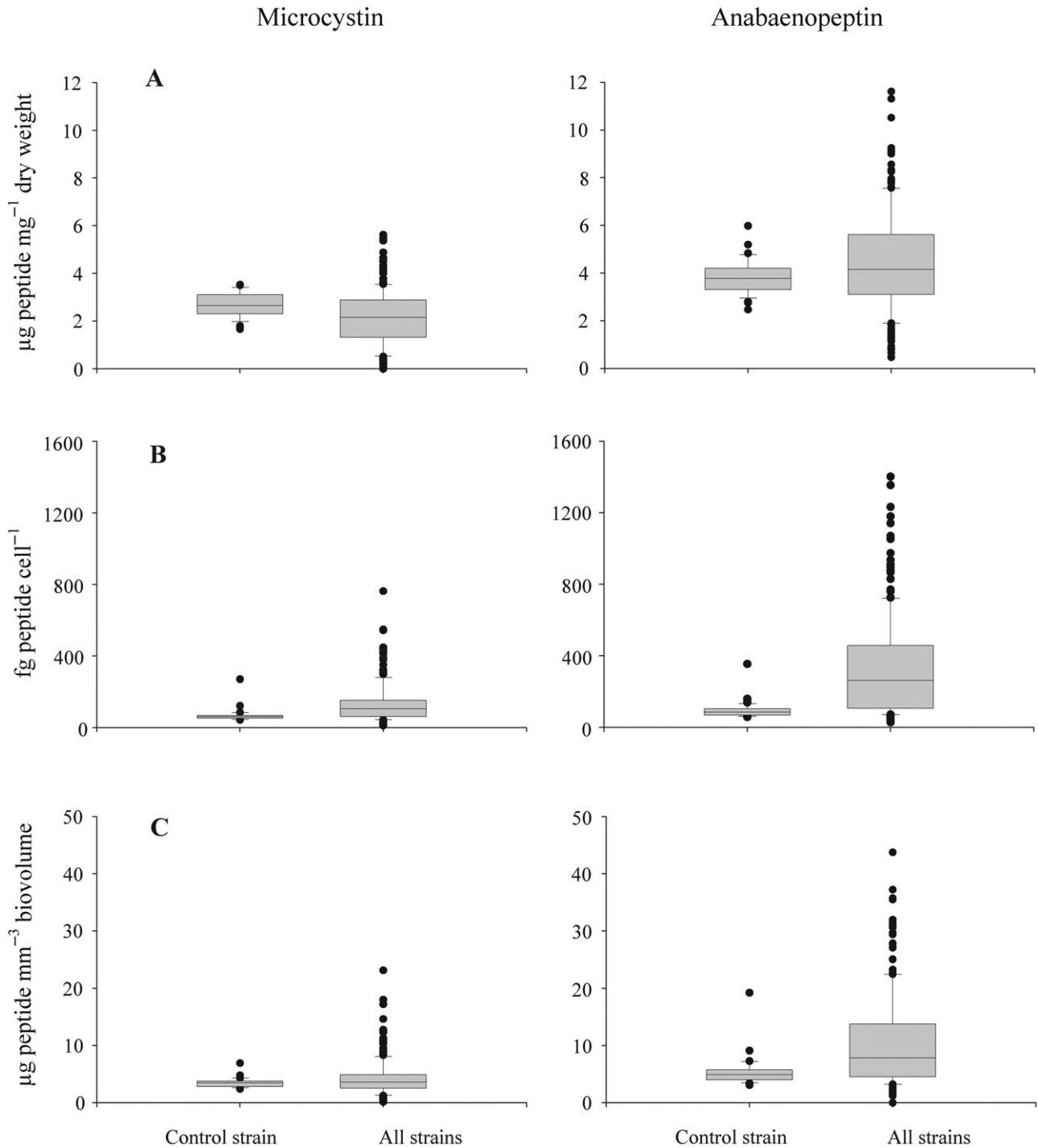


Fig. 2. Variability of microcystin (MC) and anabaenoheptin (APN) contents within the control strain *P. agardhii* No. 32 and between strains of *P. agardhii* and *P. rubescens* ($n = 49$). The control strain was used as a reference during all experiments to standardize for within strain variation. MC/APN content per (A) dry weight (three outliers of APN contents not shown), (B) cells and (C) biovolume (two outliers of APN contents not shown).

to vary between zero (nine strains) and $4.5 \pm 0.4 \mu\text{g mg}^{-1}$ DW (No. 82). In contrast the APN content differed significantly between species (*P. agardhii*: mean \pm SE, $2.9 \pm 0.4 \mu\text{g mg}^{-1}$ DW; *P. rubescens*: $5.0 \pm 0.4 \mu\text{g mg}^{-1}$ DW; t -test, $p < 0.001$), while the APN content ranged from zero (two strains) to $7.2 \pm 0.7 \mu\text{g mg}^{-1}$ DW (No. 66) in *P. agardhii* and from zero (CCAP 1459/14) to $10.3 \pm 1.3 \mu\text{g mg}^{-1}$ DW (No. 64, Table 1) in *P. rubescens*.

In general, strains contained one or two structural variants of MC, in two strains of *P. agardhii* three variants were found (Table 2). Strains either contained [Asp, Mdha]-MC-RR/[Asp, Dhb]-MC-RR only or [Asp, Mdha]-MC-RR/[Asp, Dhb]-MC-RR paired with lower amounts of [Asp]-MC-LR, and [Asp]-MC-HtyR. A number of strains did not contain any MC-RR but contained [Asp]-MC-HtyR paired with [Asp]-MC-LR. The new MCs [Asp, Dhb]-MC-HtyY

Table 1. Strains of *P. agardhii* and *P. rubescens* analysed for their microcystin (MC), anabaenopeptin (APN) and chlorophyll *a* contents (mean \pm ISE), estimated per dry weight (DW), per cell and per mm³ of biovolume.

Strain	Average filament diameter (μm)	Microcystin					Anabaenopeptin					Chlorophyll <i>a</i>				
		$\mu\text{g mg}^{-1}$ DW	Fg cell ⁻¹	$\mu\text{g mm}^{-3}$ biovolume	Dissolved MC%	$\mu\text{g mg}^{-1}$ DW	Fg cell ⁻¹	$\mu\text{g mm}^{-3}$ biovolume	Dissolved APN%	$\mu\text{g mg}^{-1}$ DW	Pg cell ⁻¹	$\mu\text{g mm}^{-3}$ biovolume	<i>n</i> ^c			
<i>P. agardhii</i>																
No. 28/2	3.0 \pm 0	0	0	0	0	0.85 \pm 0.1	39 \pm 6	1.65 \pm 0.2	nd	16.7 \pm 1.2	0.77	33.6 \pm 0.1	4			
No. 66	3.2 \pm 0.1	0	0	0	7.23 \pm 0.9	248 \pm 42	10.3 \pm 1.4	1.7 \pm 0.3	15.5 \pm 3	0.52 \pm 0.1	21.13 \pm 4.4	3				
CCAP 1459/36 ^b	3.1 \pm 0	0	0	0	2.42 \pm 0.3	99 \pm 8	3.91 \pm 0.3	0	10.7 \pm 0.2	0.51 \pm 0	20.64 \pm 0.7	4				
PCC 7811	3.2 \pm 0	0	0	0	1.8 \pm 0.8	117 \pm 27	4.41 \pm 0.9	33 \pm 15	nd	nd	nd	4				
PCC 7805	2.7 \pm 0	0	0	0	0	0	0	0	16.9 \pm 2.2	0.62 \pm 0	33.12 \pm 0.3	4				
No. 70/1a	2.8 \pm 0	1.17 \pm 0.1	44 \pm 29	2.42 \pm 1.5	62 \pm 23	2.05 \pm 0.2	67 \pm 34	3.75 \pm 1.7	58 \pm 22	0.64 \pm 0	36.6 \pm 0.2	2				
CCAP 1459/21	5.3 \pm 0.1	1.34 \pm 0.1	124 \pm 16	1.89 \pm 0.2	19 \pm 1.9	4.07 \pm 0.3	371 \pm 43	5.68 \pm 0.6	5.8 \pm 0.6	nd	nd	4				
CCAP 1459/16	5.0 \pm 0.1	1.57 \pm 0.1	148 \pm 17	2.3 \pm 0.2	0	3.65 \pm 0.3	323 \pm 22	5.01 \pm 0.3	0	nd	nd	4				
CCAP 1460/5	4.4 \pm 0.1	1.81 \pm 0.1	123 \pm 28	2.73 \pm 0.7	2.9 \pm 1.4	4.46 \pm 0.4	325 \pm 111	7.29 \pm 2.7	0.6 \pm 0.4	nd	nd	6				
CYA 126/8	2.4 \pm 0	1.93 \pm 0.4	147 \pm 8	9.7 \pm 0.5	3.8 \pm 0.6	2.13 \pm 0.1	209 \pm 67	13.79 \pm 4.4	8.0 \pm 1.2	0.28	19.16	4				
No. 70	2.9 \pm 0.1	1.96 \pm 0.2	312 \pm 127	16.7 \pm 6.4	5.2 \pm 0.1	2.97 \pm 0.1	453 \pm 129	24.32 \pm 6.3	3.7 \pm 0.2	nd	nd	2				
CCAP 1459/11A	5.5 \pm 0.2	2.0 \pm 0.3	343 \pm 73	4.8 \pm 1.1	20 \pm 3	0	0	0	0	1.62 \pm 0.4	22.4 \pm 5.2	4				
No. 32 ^a	2.9 \pm 0	2.68 \pm 0.1	67 \pm 6	3.78 \pm 0.3	5.7 \pm 1.3	3.83 \pm 0.1	96 \pm 8	5.41 \pm 0.4	0.9 \pm 0.5	12.5 \pm 1.0	18.22 \pm 2.2	38				
No. 79	3.0 \pm 0	2.75 \pm 0.1	80 \pm 4	3.54 \pm 0.2	9.4 \pm 3.7	4.05 \pm 0.7	116 \pm 15	5.1 \pm 0.6	7.8 \pm 2.4	0.33 \pm 0.1	14.87 \pm 2.4	4				
No. 79/11	2.8 \pm 0	2.77 \pm 0.3	91 \pm 14	5.09 \pm 0.8	14 \pm 6	1.78 \pm 0.4	56 \pm 9	3.13 \pm 0.5	11.4 \pm 4.6	nd	nd	4				
No. 39	3.0 \pm 0	3.19 \pm 0.2	93 \pm 9	4.75 \pm 0.4	8.0 \pm 0.4	4.75 \pm 0.2	141 \pm 17	7.19 \pm 0.7	8.3 \pm 1.7	nd	nd	4				
SAG 6.89	3.3 \pm 0	3.79 \pm 0.9	135 \pm 8	4.96 \pm 0.3	5.3 \pm 3.6	2.73 \pm 0.8	119 \pm 36	4.38 \pm 1.3	0	0.33 \pm 0	12.34	4				
No. 31/1	2.7 \pm 0	4.46 \pm 0.4	117 \pm 9	7.03 \pm 0.5	10.5 \pm 0.9	4.0 \pm 0.5	106 \pm 17	6.35 \pm 0.9	24 \pm 2.4	nd	nd	4				
<i>P. rubescens</i>																
No. 10	3.1 \pm 0	0	0	0	0	9.75 \pm 1.7	613 \pm 194	27.37 \pm 8.7	2.5 \pm 1.4	10.2 \pm 2.2	0.68 \pm 0.1	29 \pm 7	4			
No. 12/1a	3.6 \pm 0.1	0	0	0	0	4.48 \pm 0.3	333 \pm 63	11.82 \pm 2.2	33 \pm 6.5	6.1 \pm 0.9	16.6 \pm 2.4	4				
No. 40 ^b	4.1 \pm 0.1	0	0	0	0	3.55 \pm 0.3	701 \pm 334	15.48 \pm 7	0	8.4 \pm 0.1	57.6 \pm 37.6	4				

No. 65	3.6 ± 0.1	0	0	0	4.7 ± 1.2	300 ± 56	10.13 ± 1.8	33 ± 4.3	5.9 ± 0.3	0.61 ± 0.4	20.4 ± 12.5	4
No. 67 ^b	3.0 ± 0	0	0	0	7.02 ± 2.2	772 ± 223	31.36 ± 8.7	3.2 ± 3.2	7.2 ± 1.5	1.0 ± 0.2	40.5 ± 13.7	4
No. 91/1 ^b	3.7 ± 0.1	0	0	0	5.34 ± 0.6	241 ± 48	14.81 ± 5	3.4 ± 1.7	9.5 ± 0.1	0.4 ± 0.3	18.7 ± 2.7	4
No. 110 ^b	3.3 ± 0.1	0	0	0	4.71 ± 0.5	276 ± 45	10.62 ± 1.6	4.0 ± 1.1	nd	nd	nd	4
No. 119	3.6 ± 0.1	0	0	0	7.1 ± 0.9	604 ± 153	17.46 ± 4.5	2.5 ± 2.2	10.2 ± 0	1.11 ± 0.3	32.1 ± 9.6	4
No. 160/1a	4.9 ± 0.1	0	0	0	2.56 ± 0.2	263 ± 125	4.18 ± 1.8	16 ± 2	nd	nd	nd	2
PCC 7821	2.9 ± 0.1	0.32 ± 0.1	28 ± 3	1.23 ± 0.1	3.3 ± 0.6	295 ± 51	12.89 ± 2.2	5.5 ± 0.3	4.8 ± 1.6	0.58	25.22	4
No. 59	5.0 ± 0.1	0.33 ± 0.1	34 ± 12	0.51 ± 0.2	3.91 ± 0.4	472 ± 150	7.04 ± 2.1	5.9 ± 1	nd	nd	nd	4
No. 60	4.8 ± 0.1	0.37 ± 0	34 ± 5	0.65 ± 0.1	3.96 ± 0.2	390 ± 82	7.41 ± 1.7	2.0 ± 1.3	5.6 ± 1.2	0.45 ± 0.1	8.24 ± 1.3	4
No. 14/1a	3.4 ± 0.1	0.53 ± 0	30 ± 6	1.18 ± 0.3	4.89 ± 0.1	262 ± 28	10.36 ± 1.3	2.7 ± 1	nd	nd	nd	4
No. 80	3.3 ± 0.1	0.58 ± 0	27 ± 6	1.11 ± 0.3	5.85 ± 0.3	277 ± 83	11.48 ± 3.7	2.2 ± 2.2	6.0 ± 1.3	0.3 ± 0	12.62 ± 1.3	2
No. 108	5.4 ± 0.1	0.71 ± 0	nd	nd	2.01 ± 0.1	nd	nd	28 ± 11	nd	nd	nd	2
No. 21/2	4.2 ± 0.1	0.78 ± 0.2	100 ± 25	2.29 ± 0.5	2.1 ± 1.3	4.07 ± 0.1	11.29 ± 1.5	2.1 ± 0.7	nd	nd	nd	4
No. 90/1i	3.2 ± 0.1	0.84 ± 0.2	105 ± 26	4.85 ± 1.2	5.66 ± 0.9	728 ± 191	33.47 ± 8.4	27 ± 1	nd	nd	nd	4
No. 64	4.2 ± 0.1	0.89 ± 0.1	101 ± 11	2.15 ± 0.1	4.5 ± 1.7	1156 ± 129	24.66 ± 1.7	3.5 ± 0.2	10.1 ± 0.1	1.12 ± 0.0	24.1 ± 0.8	4
CCAP 1459/24	4.7 ± 0.1	1.36 ± 0.4	219 ± 33	3.88 ± 0.6	5.5 ± 1.3	390 ± 58	6.9 ± 1	0	8.2 ± 1	1.55 ± 0.3	28.2 ± 5.7	2
No. 72	3.4 ± 0.1	1.41 ± 0.1	87 ± 4	3.45 ± 0.1	2.7 ± 0.2	527 ± 38	21.47 ± 2.2	2.3 ± 0.4	nd	nd	nd	4
No. 118	4.3 ± 0.1	1.47 ± 0.2	212 ± 23	4.32 ± 0.5	6.3 ± 1.8	950 ± 120	19.45 ± 2.8	4.0 ± 1.2	nd	nd	nd	3
No. 241	4.1 ± 0.1	1.40 ± 0.2	113 ± 9	2.73 ± 0.3	4.2 ± 0.4	536 ± 19	12.95 ± 0.9	0	8.9 ± 1	0.8 ± 0.1	18.0 ± 1.7	3
No. 240	4.5 ± 0.1	1.69 ± 0.1	177 ± 31	3.53 ± 0.6	4.2 ± 2.4	484 ± 118	9.68 ± 2.2	2.8 ± 2.8	nd	nd	nd	4
No. 111	3.4 ± 0.1	2.21 ± 0.2	225 ± 76	8.38 ± 3	3.1 ± 0.5	798 ± 335	29.83 ± 13.2	1.8 ± 1.1	7.5 ± 0.4	1.07 ± 0.6	40.3 ± 23.6	4
No. 3	3.4 ± 0.1	2.25 ± 0.2	136 ± 6	4.75 ± 0.2	4.6 ± 2.0	420 ± 79	14.73 ± 2.9	7.0 ± 3.1	nd	nd	nd	4
No. 113/1b	3.8 ± 0	2.36 ± 0.4	105 ± 12	3.1 ± 0.4	5.93 ± 0.6	322 ± 122	9.53 ± 3.7	4.9 ± 1.1	nd	nd	nd	4
No. 247	3.6 ± 0.1	2.41 ± 0.3	116 ± 17	3.78 ± 0.5	4.2 ± 0.3	95 ± 14	3.09 ± 0.4	6.2 ± 1.1	7.2 ± 1.5	0.36 ± 0	12.2 ± 0.1	4
CCAP 1459/14	4.1 ± 0.1	2.7 ± 0.2	212 ± 78	5.71 ± 2.0	6.1 ± 0.3	0	0	0	9.2 ± 0.9	0.44 ± 0.3	12.4 ± 7.9	4
No. 21-	4.2 ± 0	2.92 ± 0.4	334 ± 25	7.18 ± 0.6	2.8 ± 0.6	463 ± 109	9.86 ± 2.3	0	10.8 ± 1.8	1.29 ± 0.1	27.6 ± 2.2	2
CCAP 1459/30	3.2 ± 0.1	3.38 ± 0.6	185 ± 49	7.42 ± 2.0	4.0 ± 0.9	426 ± 180	17.3 ± 7.2	6.5 ± 2	nd	nd	nd	4
No. 82	4.0 ± 0.1	4.51 ± 0.4	857 ± 399	20.6 ± 9.2	12.3 ± 5.1	442 ± 170	10.9 ± 4	10.5 ± 9.2	9.0 ± 0.3	1.05	23.56	4

Notes: Within species the strains were ordered according to the amount of MC produced (second column, µg MC per mg DW). nd: not determined.

^aStrain No. 32 was included as a control when performing the growth experiments with all the other strains.

^bStrains containing the microcystin biosynthesis gene cluster which became inactive due to mutations, for example due to the insertion of mobile elements (Christiansen *et al.*, 2006).

^c*n*, number of parallels for MC/APN measurements.

Table 2. Proportion of intracellular microcystin (MC) and anabaenopeptin (APN) variants (mean ± 1 SE) in *P. agardhii* and *P. rubescens* strains.

Strain	MC variants (%)						APN variants (%)					
	[Asp, Mdha]- MC-RR ^a	[Asp, Dhb]-MC-RR ^b	[Asp]-MC-HtyR ^c	[Asp]-MC-LR ^d	[Asp, Dhb]- MC-HtyY/Hty ^{e,f}	APN B ^g	APN J ^h	APN F ⁱ	APN A ^j	APN I ^k	OSC Y ^l	
<i>P. agardhii</i>												
No. 28/2	0	0	0	0	0	100 \pm 0	0	0	0	0	0	
No. 66	0	0	0	0	0	63.7 \pm 5.5	0	36.3 \pm 5.5	0	0	0	
CCAP 1459/36	0	0	0	0	0	22.0 \pm 2.1	0	32.5 \pm 0.6	17.0 \pm 0.9	0	28.6 \pm 2.2	
PCC 7811	0	0	0	0	0	13.1 \pm 0.7	0	35.2 \pm 1.6	12.9 \pm 1.3	0	38.7 \pm 1.6	
PCC 7805	0	0	0	0	0	0	0	0	0	0	0	
No. 70/1a	0	91.0 \pm 9.0	0	9.0 \pm 9.0	0	34.3 \pm 0.7	0	32.6 \pm 6.9	33.1 \pm 6.2	0	0	
CCAP 1459/21	0	100 \pm 0	0	0	0	90.3 \pm 0.3	0	0	7.9 \pm 0.3	0	1.8 \pm 0.2	
CCAP 1459/16	0	0	71.7 \pm 1.7	28.3 \pm 1.7	0	89.1 \pm 1.4	0	0	9.7 \pm 0.7	0	1.1 \pm 0.7	
CCAP 1460/5	0	0	76.0 \pm 1.8	24.0 \pm 1.8	0	86.1 \pm 0.5	0	0.3 \pm 0.2	11.5 \pm 0.6	0	2.2 \pm 0.5	
CYA 126/8	71.0 \pm 1.7	0	0	29.0 \pm 1.7	0	0	37.9 \pm 1.6	0	0	62.1 \pm 1.6	0	
No. 70	0	84.2 \pm 0.6	0	15.8 \pm 0.6	0	33.3 \pm 5.0	0	35.3 \pm 8.9	31.3 \pm 3.9	0	0	
CCAP 1459/11A	0	100 \pm 0	0	0	0	0	0	0	0	0	0	
No. 32	73.7 \pm 0.3	0	0	26.3 \pm 0.3	0	24.9 \pm 1.1	0	44.2 \pm 2.0	27.9 \pm 0.9	0	3.0 \pm 0.3	
No. 79	84.8 \pm 0.6	0	0	15.2 \pm 0.6	0	16.1 \pm 1.9	0	4.3 \pm 0.4	28.3 \pm 5.3	0	51.3 \pm 5.6	
No. 79/11	82.2 \pm 0.7	0	0	17.8 \pm 0.7	0	28.8 \pm 0.9	0	2.1 \pm 2.1	69.1 \pm 1.5	0	0	
No. 39	72.8 \pm 0.2	0	0	27.4 \pm 0.2	0	28.9 \pm 1.0	0	71.1 \pm 1.0	0	0	0	
SAG 6.89	73.4 \pm 0.2	0	1.5 \pm 0.8	25.2 \pm 0.9	0	16.8 \pm 4.4	0	29.6 \pm 1.9	14.7 \pm 0.3	0	39.0 \pm 6.5	
No. 31/1	65.5 \pm 1.1	0	3.0 \pm 0.4	31.4 \pm 1.2	0	8.3 \pm 0.7	0	30.9 \pm 1.0	16.8 \pm 1.1	0	44.0 \pm 1.9	
<i>P. rubescens</i>												
No. 10	0	0	0	0	0	40.0 \pm 1.8	0	16.1 \pm 0.9	26.7 \pm 2.1	0	17.2 \pm 0.6	
No. 12/1a	0	0	0	0	0	35.5 \pm 0.7	0	23.1 \pm 2.0	20.8 \pm 1.1	0	20.6 \pm 1.2	
No. 40	0	0	0	0	0	32.2 \pm 1.4	0	67.8 \pm 1.4	0	0	0	
No. 65	0	0	0	0	0	35.2 \pm 1.1	0	26.2 \pm 1.3	17.3 \pm 1.7	0	21.3 \pm 1.0	
No. 67	0	0	0	0	0	74.2 \pm 0.3	0	0.8 \pm 0.1	25.0 \pm 0.3	0	0	

No. 91/1	0	0	0	0	29.8 ± 0.4	0	21.6 ± 0.6	26.1 ± 0.8	0	22.5 ± 0.4
No. 110	0	0	0	0	53.6 ± 3.1	0	20.8 ± 1.2	8.7 ± 0.5	0	16.9 ± 1.6
No. 119	0	0	0	0	36.0 ± 0.6	0	19.2 ± 1.3	24.0 ± 1.1	0	20.8 ± 0.8
No. 160/1a	0	0	0	0	14.5 ± 0.5	0	85.4 ± 0.5	0	0	0
PCC 7821	0	100 ± 0	0	0	5.6 ± 0.4	0	12.2 ± 1.1	20.9 ± 1.9	0	61.2 ± 1.7
No. 59	0	0	0	100 ± 0	100 ± 0	0	0	0	0	0
No. 60	0	0	0	100 ± 0	99.6 ± 0.4	0	0.4 ± 0.4	0	0	0
No. 14/1a	0	0	0	100 ± 0	100 ± 0	0	0	0	0	0
No. 80	0	0	0	100 ± 0	100 ± 0	0	0	0	0	0
No. 108	0	100 ± 0	0	0	47.8 ± 2.9	0	52.2 ± 2.9	0	0	0
No. 21/2	0	0	64.2 ± 0.8	35.8 ± 0.8	37.1 ± 3.2	0	62.9 ± 3.2	0	0	0
No. 90/1i	100 ± 0	0	0	0	21.5 ± 0.6	0	26.9 ± 0.8	18.0 ± 2.0	0	33.6 ± 1.8
No. 64	100 ± 0	0	0	0	28.6 ± 1.1	0	36.9 ± 1.7	29.1 ± 2.4	0	5.5 ± 0.3
CCAP 1459/24	0	78.4 ± 2.1	0	21.6 ± 2.1	9.6 ± 1.0	0	52.0 ± 1.0	2.8 ± 0.9	0	35.6 ± 0.7
No. 72	100 ± 0	0	0	0	28.9 ± 0.6	0	71.1 ± 0.6	0	0	0
No. 118	0	80.5 ± 1.7	0	19.5 ± 1.7	6.2 ± 0.4	0	20.3 ± 0.8	19.8 ± 2.8	0	53.7 ± 2.7
No. 241	100 ± 0	0	0	0	27.5 ± 2.8	0	62.4 ± 2.8	3.5 ± 0.4	0	6.6 ± 0.6
No. 240	100 ± 0	0	0	0	34.2 ± 0.6	0	38.0 ± 1.0	10.5 ± 1.1	0	17.2 ± 0.8
No. 111	100 ± 0	0	0	0	37.6 ± 0.9	0	21.5 ± 0.9	21.3 ± 0.2	0	19.6 ± 0.3
No. 3	100 ± 0	0	0	0	37.8 ± 1.9	0	22.2 ± 1.1	19.5 ± 1.4	0	20.5 ± 0.8
No. 113/1b	100 ± 0	0	0	0	36.5 ± 0.9	0	26.0 ± 3.6	24.8 ± 1.0	0	12.7 ± 5.0
No. 247	0	79.9 ± 0.5	0	20.1 ± 0.5	12.7 ± 2.2	0	87.3 ± 2.2	0	0	0
CCAP 1459/14	0	0	63.7 ± 1.3	36.3 ± 1.3	0	0	0	0	0	0
No. 21-	0	0	49.8 ± 1.5	50.2 ± 1.5	27.0 ± 3.4	0	73.0 ± 3.4	0	0	0
CCAP 1459/30	0	39.4 ± 0.9	0	60.6 ± 0.9	3.0 ± 1.0	0	36.0 ± 6.4	8.7 ± 0.8	0	52.3 ± 6.6
No. 82	0	90.0 ± 1.1	0	9.9 ± 1.1	11.1 ± 0.5	0	22.3 ± 3.8	16.6 ± 3.1	0	50.0 ± 1.5

^a[D-Asp, Mdha]-MC-RR, *m/z* 1024, ^b[D-Asp, Dhbj]-MC-RR *m/z* 1024; ^cMC-HtyR *m/z* 1045; ^dMC-LR *m/z* 995; ^e[Asp, Dhbj]-MC-HtyY, *m/z* 1074; ^f[Asp, Dhbj]-MC-HtyHty, *m/z* 1088; ^gAPN B *m/z* 836; ^hAPN J *m/z* 909; ⁱAPN F *m/z* 850; ^jAPN A *m/z* 843; ^kAPN I *m/z* 916; ^lOSC Y *m/z* 858.

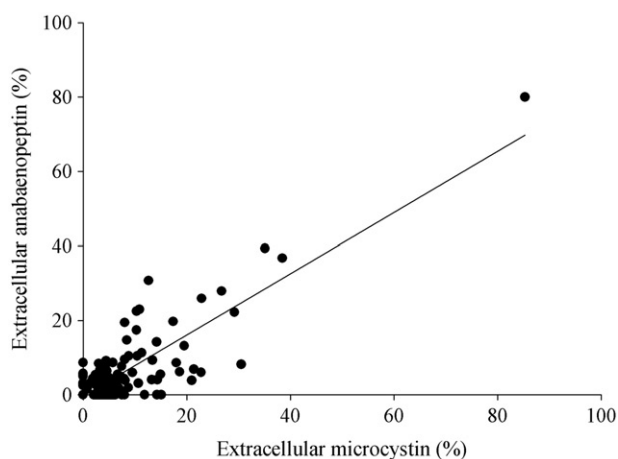


Fig. 3. Correlation of the percentages of extracellular microcystin and anabaenopeptin concentrations in *Planktothrix* strains ($R^2 = 0.77$).

and [Asp, Dhb]-MC-HtyHty were never paired with the other MC variants.

Among APNs six different variants were observed (Table 2). The majority of the strains (25 strains) contained APN B, A, F and OSC Y. A few strains contained APN B only (four strains). Ten strains produced two APN variants (APN B and F), while six strains produced three APN variants [APN B, F, A (8%), APN B, A, OSC Y (4%)]. APN I and J were found in strain CYA 126/8 only.

Extracellular peptide concentrations

All MC-producing *Planktothrix* sp. strains were analysed for extracellular MCs in the culture media ($n = 144$). Seventy seven % of the samples were found to contain less than 10% dissolved MCs of total MC content, 91% of the samples contained less than 20% dissolved MCs. On average $8.8 \pm 1.9\%$ of MC were found extracellular. A few strains (No. 70/1a, No. 108, No. 90/1i, CCAP 1459/11A, CCAP 1459/21) showed a higher proportion of dissolved MCs.

As for the MCs, the majority of the APN-producing strains ($n = 168$) contained less than 10% of the dissolved fraction (83%). In 90% of the strains the percentage of the dissolved fraction was $< 20\%$. On average $8.4 \pm 1.8\%$ of APN were found extracellular. The amount of extracellular MC and APN showed a significant positive correlation: $y = 0.82x - 0.36$ ($R^2 = 0.77$, Fig. 3). The acid ratio as determined for the chlorophyll *a* content was used to infer the physiological status and a possible peptide leakage of deteriorating cells in the cultures. Some strains (No. 70/1a, No. 82) showed high extracellular peptide concentrations paired with a high $A/A_a > 1.7$ and no correlation was found between the amount of extracellular

peptide concentration and the acid ratio. Therefore a passive leakage of MC and APN in consequence to cell death was considered to be of minor importance.

Microcystin content in dependence on the anabaenopeptin content

The majority of the strains (41/49) contained significantly higher amounts of APN when compared with MC (Mann-Whitney rank sum test, $p < 0.001$). One strain produced neither MC nor APN, five strains contained equal amounts of MC and APN (no statistically significant difference), while two strains contained MC only. With the exception of strain PCC 7805 all strains without detectable MC contained APN.

MC-producing strains were divided into five groups according to their MC content (Fig. 4). Those groups were found to differ significantly in MC content (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p < 0.001$). The APN content (intracellular and extracellular) varied independently from the MC content (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p = 0.62$).

Cellular growth rate and peptide net production rate

The growth rates among all the strains varied 5-fold as calculated from dry weight (min-mean \pm SE-max: $0.07 - 0.2 \pm 0.01 - 0.33$), however, 21-fold as calculated from cell numbers ($0.03 - 0.29 \pm 0.02 - 0.64$) and 24-fold as calculated from biovolume ($0.03 - 0.29 \pm 0.02 - 0.64$). The growth rates of the 18 *P. agardhii* strains (DW: $0.07 - 0.18 \pm 0.01 - 0.28$; cell numbers: $0.11 - 0.27 \pm 0.03 - 0.64$; biovolume: $0.12 - 0.27 \pm 0.03 - 0.64$) did not differ significantly from the growth rates as measured for the 31 *P. rubescens* strains (DW: $0.13 - 0.20 \pm 0.01 - 0.33$; cell numbers: $0.03 - 0.30 \pm 0.02 - 0.6$; biovolume: $0.03 - 0.30 \pm 0.02 - 0.61$).

The MC and APN net production rates varied up to 19-fold (min-mean \pm SE-max, MC: $0.02 - 0.25 \pm 0.01 - 0.42$, APN: $0.03 - 0.18 \pm 0.01 - 0.66$). There was no difference found between the peptide net production rates of *P. agardhii* and *P. rubescens*. Among all strains the peptide net production rates varied independently from the variation in cellular growth rates (Fig. 5), i.e. fast growing strains did not show higher peptide production rates when compared with slow growing strains or *vice versa*. There was also no difference found between the growth rates of MC/APN-producing strains and those containing no MC/APN.

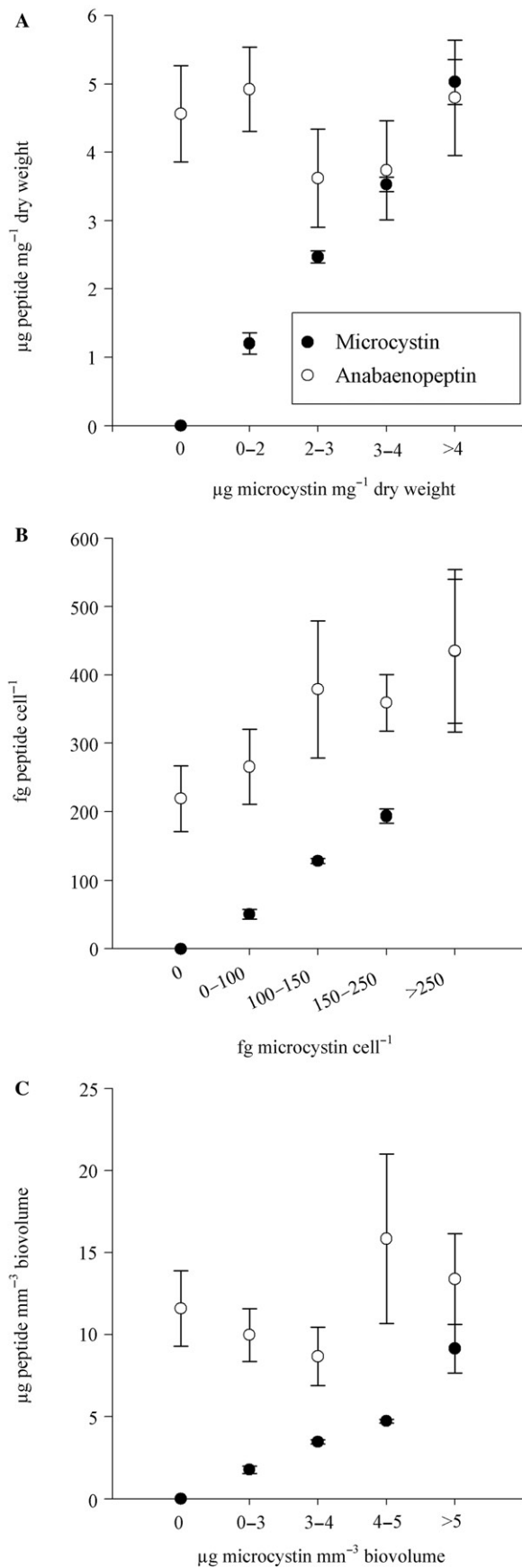


Fig. 4. Total microcystin and anabaenopeptin contents (mean \pm SE) in (A) $\mu\text{g mg}^{-1}$ dry weight, (B) fg peptide cell⁻¹ and (C) $\mu\text{g mm}^{-3}$ biovolume of *Planktothrix* strains, divided into five groups according to their microcystin contents.

Discussion

In this study an attempt was made to find a relationship between peptide net production and cell division rates between strains. The growth conditions at the temperature and light conditions used in this study (20°C , $40\text{--}60 \mu\text{mol m}^{-2} \text{s}^{-1}$) were not optimal for all the strains and a significant intraspecific variation in maximal-specific growth rates has been observed. While such an intraspecific variation has been found earlier (Van Liere & Mur, 1980; Davis & Walsby, 2002) the general understanding of factors causing this intraspecific variation in growth under identical experimental conditions in the laboratory is not complete. Mikkola & Kurland (1992) explained a threefold intraspecific variation in growth rates ($0.48\text{--}1.43$ doublings h^{-1}) among natural isolates of *Escherichia coli* by strain-specific differences in translational efficiency. The same authors were able to select for highest growth rates (1.33 doublings h^{-1}) under continuous culture conditions over 280 generations implying that selection for faster growth rates under nutrient poor conditions in nature is of minor importance when compared with the nutrient rich conditions in the laboratory.

However, to accept or to reject the hypothesis that the growth rate is related to peptide net production not only within a strain but between strains it is not necessary to find out the maximum intrinsic growth rate for each strain. According to the conclusions proposed by Orr & Jones (1998) a slow growing strain can be expected to show an increase in peptide net production if the real maximum intrinsic growth rate would have been found. In contrast to the results observed by Orr & Jones (1998), in this study a linear relationship between the peptide net production rate and the cellular growth rate obtained from all strains could not be found. Indeed strains growing fast had both low and high MC and APN contents and MC and APN production rates (Fig. 5). It is known that NRPS are large multi-enzyme complexes synthesizing peptides by the thiotemplate-directed mechanism, independent of the ribosomal peptide synthesis pathway (Marahiel *et al.*, 1997). Typically, secondary metabolites such as antibiotics synthesized via NRPS are then actively exported out of the cell and thought to enhance the competitiveness or confer resistance to the producer itself (see Pearson *et al.*, 2004 for a review). In contrast for MCs export has not been observed (Wiedner *et al.*, 2003; Tonk *et al.*, 2005; Rohrlack & Hyenstrand, 2007) and higher extracellular concentrations have only been reported from dense cultures making it impossible to differentiate between passive leakage and active export of

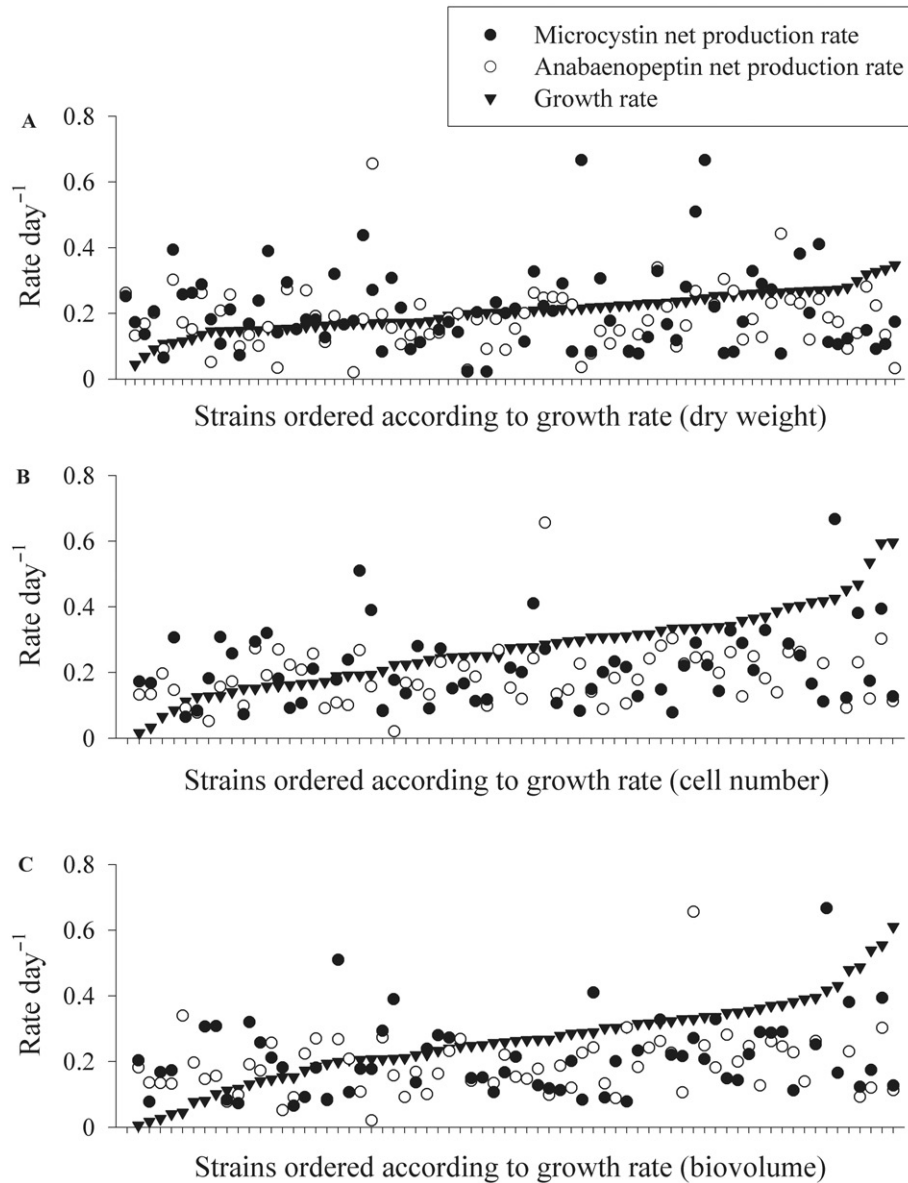


Fig. 5. Growth rates (black triangles) and corresponding peptide production rates (microcystin, black circles and anabaenopeptin, white circles) for 49 *Planktothrix* strains measured under maximum growth rate conditions. Cell growth rates were estimated from (A) dry weight, (B) cell numbers and (C) biovolume.

MC. Accordingly in this study the average dissolved peptide concentrations were found to make up less than 10% of the total peptide contents. Instead cyanobacteria seem to accumulate MC and other peptides in certain cell regions, i.e. immunogold labelling revealed preferential localization of MCs in thylakoids and in the periphery of polyphosphate bodies (Shi *et al.*, 1995; Young *et al.*, 2005). Recently a binding of MCs and cyanopeptolins to phycobiliproteins situated onto the thylakoid membranes has been reported (Jüttner & Lüthi, 2008). It is concluded that the frequently observed relationship between cell division and MC net production rate is rather the result of an accumulation of MCs in specific cell compartments rather than due to a direct dependence of MC synthesis on the processes involved in the cell-division cycle.

From the results of this study it follows that the variation in the cellular growth rate is independent from the increase/decrease of the MC/APN production. Indeed the insertional inactivation of the MC synthesis by a gene knock-out experiment in *Microcystis* did not lead to a measurable difference in growth rate under various light conditions ($4\text{--}110\ \mu\text{mol m}^{-2}\text{s}^{-1}$) as compared with the wild type (Hesse *et al.*, 2001). Correspondingly, the knock-out mutant lacking anabaenopeptilide in *Anabaena* strain 90 did not show increased growth rates of the mutant when compared with the wild type (Repka *et al.*, 2004). In this study, several strains containing the total *mcy* gene cluster (CCAP 1459/36, No. 40, No. 67, No. 91/1, No. 110) but inactive in MC synthesis due to the insertion of a transposable element (Christiansen *et al.*, 2006), did not show higher growth rates when

compared with the other MC-producing strains. Consequently, costs related to MC production and of other NRS peptides at the post-translational level should not be considered to be a factor influencing cellular growth rates and potential competitive replacement between MC-producing and non-MC-producing strains. Rather the deletion of NRPS gene clusters or larger parts of it may provide sufficient physiological advantage by reducing the genome size, as the selection coefficient on small deletions is considered negligible (Mira *et al.*, 2001). In fact subcultures of strains of *Microcystis* that lost a larger part of the MC synthesis gene cluster (>34 kbp) have been observed to succeed the MC-producing original strain (Schatz *et al.*, 2005).

In summary, peptide contents and growth rates between strains are highly variable and independent of each other. No relationship of MC net production with regard to the life-form of both species *P. agardhii* and *P. rubescens* could be found, implying that MC is not directly related with the ecological divergence observed (Reynolds *et al.*, 2002). Most studies so far were based on the genus *Microcystis*, however, as argued by one referee, there is no reason to assume principal differences between the two genera with regard to the results obtained in this study. For the purpose of comparing estimates of MC cell contents between partners of the EU-project PEPCY (www.pepcy.de, Nov 2002–June 2006), *Microcystis* strain PCC 7806 has been analysed when grown in O₂ medium (Van Liere & Mur 1978) but under otherwise identical conditions as for *Planktothrix*. For three parallels the results were $3.6 \pm 0.3 \mu\text{g MC mg}^{-1} \text{ DW}$, $71 \pm 12 \text{ fg MC cell}^{-1}$, $1.9 \pm 0.3 \mu\text{g MC mm}^{-3} \text{ biovolume}$ with $10 \pm 1.4\%$ of dissolved MC. This result compares with other studies on *Microcystis* cellular MC contents (Wiedner *et al.*, 2003, Saker *et al.*, 2005).

It is emphasized that the intraspecific differences in cellular growth rate have been observed in nutrient-rich media in the laboratory and the relevance of those differences in strain-specific growth rates under natural conditions has not been documented. To date, the influence of strain-specific growth rates on the varying abundance of MC-producing and non-MC-producing genotypes in the environment remains speculative (Sivonen & Jones, 1999) and aside from genotype-specific growth rates as a consequence of ecological divergence other factors such as genotype-specific losses due to bacteriophages or algae-lysing bacteria need to be considered. First results, however, rather point to a coexistence of genotypes differing in peptide production that can last for years (Christiansen *et al.*, 2006) or even decades (Guljamow *et al.*, 2007).

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