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Experimental comparison of phenotypical plasticity and growth demands of two strains from the *Anabaena circinalis/A. crassa* complex (cyanobacteria)

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Two cyanobacterial strains were isolated in 2004 from different localities in the Czech Republic. Field morphology of the strain 04-26 (Jesenice reservoir) matched with the species description of Anabaena crassa (Lemm.) Kom.-Legn. et Cronb. 1992, whereas the strain 04-28 (Hodějovický fishpond) was identified as A. circinalis Rabenh. ex Born. et Flah. 1888. Both these strains, exposed to various experimental conditions (temperature, light intensity, nitrogen and phosphorus concentration), displayed highly similar morphologies and spanned the morphological variability of both of the above-mentioned species. Significant relationships between environmental conditions (temperature, phosphorus) and morphological characteristics (vegetative cell and heterocyte dimensions, trichome coiling parameters) have been recorded for the first time within the genus Anabaena. The strains studied differed in their temperature and light growth optima and in secondary metabolite contents. However, both were identical (100% similarity) in their 16S rRNA gene sequence and showed 99.9–100% similarity to the published 16S rRNA sequences of A. circinalis strains from northern Europe.

INTRODUCTION

The planktonic cyanobacterium *Anabaena circinalis* Rabenh. ex Born. et Flah. 1888 is a common morphospecies that has been reported from all continents and its blooms are a major worldwide problem due to the production of a wide range of toxins (Beltran and Neilan, 2000). The knowledge of its ecology, morphological variability, toxicity and phylogenetic status is therefore of particular interest.

A similar morphospecies, *A. crassa* (Lemm.) Kom.-Legn. et Cronb. 1992, was described from Sweden (Komárková-Legnerová and Cronberg, 1992) and is common in the temperate zone worldwide (Komárek, 1996; Cronberg and Annadotter, 2006). The trichome width and the diameters of trichome coils are regarded as the main distinguishing criteria between *A. crassa* and *A. circinalis* (Komárek, 1958; Komárková-Legnerová and Cronberg, 1992; Komárková-Legnerová and Eloranta, 1992; Komárek and Zapomělová, 2007). However, morphological comparison of 13 *A. circinalis* and *A. crassa* populations from the Czech Republic has demonstrated

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continuous transitions of both the trichome widths and the coil diameters (Zapomělová *et al.*, 2007).

Phylogenetic comparison of several strains of *A. circinalis* and *A. crassa* was published by Rajaniemi (Rajaniemi *et al.*, 2005a, b). The results were consistent with the above-mentioned morphological study (Zapomělová *et al.*, 2007), since both the morphospecies clustered closely together, based on 16S rRNA gene, *rpoB* and *rbcLX* sequences.

Ecological demands of the cyanobacteria from the *A. circinalis/A. crassa* complex, the range of their morphological variability under varying growth conditions or secondary metabolite production have not been compared so far. Consequently, the present study focuses on morphological plasticity of two strains of this cyanobacterial complex under varied conditions of temperature, light intensity and nitrogen or phosphorus concentration. The temperature and light growth optima and secondary metabolite content of these strains have been compared. In addition to morphological evaluation, the studied strains have also been characterized by partial sequences of the 16S rRNA gene.

METHOD

Sampling, isolation and cultivation

Samples of blooms were collected in August and September 2004 from two localities (Jesenice reservoir, Hodějovický fishpond) using a 20 μm mesh plankton net. Jesenice (50°5′1.88″N, 12°28′29.71″E) is a deep dimictic mesotrophic reservoir in the west of the Czech Republic. It is situated on the river Odrava and serves for recreational purposes. Hodějovický fishpond (48°56′36.63″N, 14°29′35.88″E) is a small and shallow polymictic eutrophic pond on an unnamed brook in the south of the Czech Republic. Morphology of fresh material was evaluated immediately as described below. Single trichomes were isolated from the phytoplankton samples as described by Zapomělová (Zapomělová *et al.*, 2007). Clonal cultures were grown in WC medium (Guillard and Lorenzen, 1972) at 21°C and a light intensity of 70 μmol m^{-2} s^{-1} (16:8 L:D cycle). The strain isolated from Jesenice reservoir was named 04-26 and the strain from Hodějovický fishpond 04-28.

Crossed gradients of light and temperature

Crossed gradients (Kvíderová and Lukavský, 2001) were used to test the effect of light and temperature on the cyanobacterial morphology and to determine the growth optima. For the morphological experiments, the strains were exposed in sterile culture plates (9 × 12 cm, 12 wells, 6.5 mL each) to nine different combinations of light and temperature in crossed gradients (Fig. 1a) for 10 days. The temperature ranged from 10°C to 28°C and the range of light intensity, provided by sodium-vapour lamps, was 20–750 μmol m^{-2} s^{-1}. To estimate the temperature and light growth optima of the strains, a modified design of the cross-gradient experiments was used (Fig. 1b). Identical volumes of stirred dense batch culture were inoculated into sterile culture plates containing fresh WC medium (9 × 12 cm, 6 wells, 16 mL each). The plates were then exposed to 25 positions of the cross-table. The temperature range was 6–34°C and the light intensity 20–750 μmol m^{-2} s^{-1}. The experiments were terminated in the exponential phase of growth of

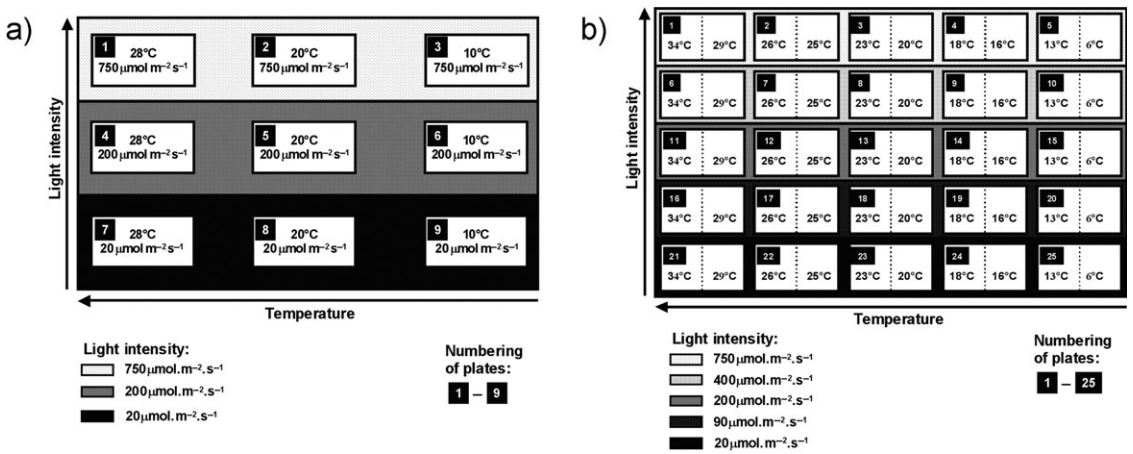

Fig. 1. Design of the cross-gradient experiments: (**a**) evaluation of morphological variability of the strains in relation to light and temperature and (**b**) determination of light and temperature growth optima of the strains. Gradients of light intensity and temperature are indicated with arrows.

the fastest growing cultures. Chlorophyll *a* concentrations were determined spectrophotometrically after acetone extraction (Lorenzen, 1967) and compared among the positions of the crossed gradients.

Concentration series of nitrogen and phosphorus

Modified types of WC medium containing different concentrations of nitrogen (N) and phosphorus (P) (Table I) were used to test the effect of nutrients on cyanobacterial morphology. The concentration series were designed with respect to N and P concentrations commonly occurring in fishponds and reservoirs of the Czech Republic (Znachor *et al.*, 2006). In order to force the strains to deplete their intracellular nutrient reserves, cyanobacterial biomass was incubated in a modified WC medium without N and P ("starving medium", Table I) for 7 days prior to the experiment. Equimolar concentrations of KCl were added to the "starving medium", $WC_{0\times P}$ $WC_{0.001\times P}$ and $WC_{0.1\times P}$ in order to retain the original K^+ concentration.

Morphometry

Microphotographs of at least 30 fresh trichomes from each field population or for each experimental treatment were taken with a digital camera (Olympus DP 70, magnification ×400). Dimensions of all cell types were measured (five vegetative cells per trichome measured in 30 trichomes and as many heterocytes and akinetes as it was possible to find in each sample). Length:width ratios of vegetative cells, heterocytes and akinetes were computed to roughly characterize the cell shapes. Trichome coil diameters and distances between neighbouring coils were measured. Diameter:distance ratios were calculated to characterize the tightness of trichome coiling. All size measurements were performed using image analysis (Olympus DP Soft).

Table I: Modifications of WC medium used in the experiments

Medium type	P (μmol L^{-1})	N (μmol L^{-1})
$WC_{0\times P}$	0	5.7×10^2
$WC_{0.001\times P}$	2.6×10^{-2}	5.7×10^2
$WC_{0.1\times P}$	2.6×10^0	5.7×10^2
$WC_{1\times P}$=$WC_{1\times N}$=WC	2.6×10^1	5.7×10^2
$WC_{10\times P}$	2.6×10^2	5.7×10^2
$WC_{0\times N}$	2.6×10^1	0
$WC_{0.001\times N}$	2.6×10^1	5.7×10^{-1}
$WC_{0.1\times N}$	2.6×10^1	5.7×10^1
$WC_{10\times N}$	2.6×10^1	5.7×10^3
"Starving medium"	0	0

Secondary metabolite content: extract preparation and HPLC-MS analysis

Lyophilized biomass (~40 mg) was disintegrated by grinding and extracted in 2 mL of 70% methanol (MeOH) in microtubes for 30 min. The microtubes were centrifuged at 3170 g at 4°C for 15 min. Supernatant was concentrated 10 times in a rotary vacuum drier. HPLC-MS analysis was performed in order to determine the content of secondary metabolites. The extracts were analysed on a reversed phase column (Zorbax XBD C8, 46 × 150 mm, 5 μm) using a $MeOH/H_2O$ gradient with a flow rate of 0.6 mL min^{-1}. For a more effective ionization, 0.1% formic acid was added to the eluents. The extract composition was analysed with an HP 1100 Agilent mass spectrometer HP 100 MSD SL-Ion trap in positive mode. The settings were selected to cover the mass range between 50 and 2000, and the ion trap was targeted to molecular masses near 900. Automatic fragmentation of the most intensive peak was applied. The molecular ions were determined based on the presence of sodium and potassium adducts and on the distribution of isotopologues.

Phylogenetic study

The biomass was harvested in the exponential phase of growth by repeated centrifugation, during which the trichomes were washed several times with physiological solution (NaCl solution, concentration 1 g L^{-1}) to remove mucilaginous substances. The biomass samples were stored at −20°C until DNA extraction. DNA was extracted using UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). The 16S rRNA gene and ITS region were amplified with primers 16S27F and 23S30R (Taton *et al.*, 2003). Amplification was carried out as follows: 1 cycle of 5 min at 94°C; 10 cycles of 45 s at 94°C, 45 s at 57°C and 2 min at 72°C; 25 cycles of 45 s at 94°C, 45 s at 54°C and 2 min at 72°C and a final elongation step of 7 min at 72°C. PCR product was used as a template for sequencing with primers 16S27F, 23S30R (Taton *et al.*, 2003), primer cAlaR (Wilmotte *et al.*, 1994) and primer CYA781F(a) (Nübel *et al.*, 1997). Sequences were aligned in the program ARB (http://www.arb-home.de). The alignment was edited manually and ambiguous bases were removed. For the phylogenetic analysis, trees were built with the neighbour joining (NJ) method (Saitou and Nei, 1987) and the maximum parsimony (MP) algorithm in the Phylip program (Felsenstein, 2004). Five hundred bootstrap replicates were performed both for NJ and MP analyses.

Nucleotide sequences have been deposited at Gen Bank under the accession numbers AM940218 and AM940219. Besides the strains whose accession numbers are given in the phylogenetic tree, the following sequences were used for the construction of the phylogenetic trees: AJ293126, AJ630441, AJ293127, AY701569, AJ293109, AJ630418, AJ293111, AJ133154, AJ293131, AJ293124, AJ133155, AJ630408, AJ630410, AJ630412, AJ630409, AJ133151, AJ293103, AJ293104, AJ133159, AJ630424, AJ630422, AJ13156, AJ293113, AJ293106, AY196088, AJ293108, AY196087, AJ630446, AJ630457, AJ630456, AJ630458, AJ630428, AF160256, AY038033, AF516747, AY038036, AJ781144, AJ133184, AJ133181, AY701557, AY701558, AY763116, AY763117, EU076459, AF067819, AF516724, AJ582102, AY699989, EU078547, EU076458, EU078548.

Statistical analysis

The effect of light, temperature, phosphorus and nitrogen on the morphometric characteristics was tested by redundancy analysis (RDA) with forward selection. A Monte-Carlo permutation test was used for calculation of *P*-values.The data were centred and standardized. These statistical analyses were performed using the program CANOCO (Ter Braak and Šmilauer, 1998) and ordination diagrams were created using CanoDraw software (Šmilauer, 1992). Basic statistical characteristics such as average values, 25 and 75% percentiles and extreme values were computed for each morphological feature. Box–whisker plots were created by the GraphPad Prism program (GraphPad Software, San Diego California USA, www.graphpad.com). Surface plots demonstrating the light and temperature growth optima were created using the program Statistica (Anonymous, 1996).

RESULTS

Morphological plasticity under varied growth conditions

The experiments demonstrated the range of morphological characteristics of the strains 04-26 and 04-28, which was markedly greater than their morphological variability observed under field conditions (Table II). The strains displayed highly similar morphologies during the experiments, although their width of trichomes, dimensions of akinetes and trichome coiling characteristics differed in the field.

Significant effects of temperature and phosphorus concentration on the morphologies of the strains studied were confirmed by RDAs, while the effects of light intensity and nitrogen concentration were insignificant (Table III). The strongest response to varied environmental factors was observed in vegetative cells, whose dimensions were significantly influenced by the phosphorus concentration in both the strains and by the temperature in the strain 04-26 (Table III). On the contrary, no significant effect of the experimental factors on the akinete dimensions and shape was found in both the strains.

The dimensions of vegetative cells and heterocytes were largest at 28°C, especially in the strain 04-26, where the effect of the temperature was stronger (Fig. 2). However,

Table II: Summary of morphological features of the strains observed during the experiments and in the field

	Strain 04-26		Strain 04-28	
Morphological characteristics	Experiments	Field	Experiments	Field
Vegetative cells				
Length (μm)	6.0–12.0	8.2–10.6	5.5–17.0	6.4–8.6
Width (μm)	8.5–13.5	11.2–12.2	7.0–13.0	10.0–11.0
Length:width ratio	0.7–1.2	0.7–0.9	0.6–2.1	0.6–0.8
Heterocytes				
Length (μm)	7.5–13.0	10.6–12.5	8.5–12.6	10.2–12.0
Width (μm)	8.5–13.0	11.2–12.2	9.0–13.0	10.6–11.8
Length:width ratio	0.9–1.1	0.9–1.0	0.9–1.2	0.9–1.0
Akinetes				
Length (μm)	20.0–30.0	24.0–29.0	18.0–29.0	17.0–25.0
Width (μm)	12.5–18.5	16.5–18.5	13.0–18.5	12.8–15.2
Length:width ratio	1.3–1.9	1.5–1.6	1.4–2.0	1.3–1.7
Trichome coiling				
Coil diameter (μm)	35.0–62.0	45.0–56.0	15.0–110.0	40.0–52.0
Coil distance (μm)	20.0–57.0	38.0–45.0	20.0–130.0	25.0–40.0
Diameter:distance ratio	0.9–1.9	0.8–1.4	0.2–2.8	1.0–1.8

Values between 25 and 75% percentiles are shown and the outlying values were omitted.

Table III: Morphological characteristics for which a significant effect of at least one environmental factor was demonstrated by RDA

Morphological criterion	Strain	Factor	Variability explained by the model (%)	Variability explained by the factor (%)	*F*-value	*P*-value
Vegetative cell morphology (length, width, length: width ratio)	04-26	**Temperature**	52.5	**44.8**	**4.871**	**0.0260**
		Light		7.7	0.806	0.4540
		Phosphorus	80.1	**75.4**	**21.469**	**0.0080**
		Nitrogen		4.7	1.425	0.2740
	04-28	Temperature	32.9	26.5	2.524	0.1000
		Light		7.4	0.559	0.5940
		Phosphorus	75.9	**70.9**	**17.073**	**0.0260**
		Nitrogen		5.0	1.242	0.3280
Heterocyte morphology (length, width, length: width ratio)	04-26	**Temperature**	85.4	**82.8**	**28.985**	**0.0020**
		Light		2.6	0.876	0.4920
		Phosphorus	31.9	29.5	2.929	0.0640
		Nitrogen		1.0	0.070	0.9560
	04-28	Temperature	33.8	32.5	2.884	0.0720
		Light		1.6	0.100	0.8680
		Phosphorus	50.6	30.7	3.097	0.0840
		Nitrogen		19.9	1.739	0.1860
Trichome coiling (coil diameters, distances of adjacent coils, coil: distance ratio)	04-26	**Temperature**	38.9	**35.4**	**3.294**	**0.0480**
		Light		3.5	0.283	0.7680
		Phosphorus	16.2	13.7	1.112	0.3700
		Nitrogen		2.2	0.154	0.8760
	04-28	Temperature	60.8	42.0	5.076	0.0500
		Light		18.8	2.876	0.1240
		Phosphorus	20.7	11.0	0.867	0.3440
		Nitrogen		8.8	0.675	0.4120

Significant effects are given in bold.

the shape of these cells did not change throughout the temperature gradient since their length:width ratios remained constant. The vegetative cell width of both the strains reached 12–13 μm at 28°C.

On the contrary, the thinnest vegetative cells (8–9 μm) were observed in the lowest phosphorus concentrations ($WC_{0\times P}$, $WC_{0.001\times P}$) and their width increased with increasing P (Fig. 3). Together with reduced width of vegetative cells at low P concentrations, their length increased. Therefore, length:width ratios of vegetative cells were higher at lower P concentrations, i.e. the cells were obviously elongated.

Gas vesicles tended to accumulate in vegetative cells more at lower light intensities and at higher nutrient concentrations, especially P (Fig. 4).

Temperature and phosphorus concentration also affected trichome morphology. A clear decrease in coil diameter and coil distance was observed at the lowest temperature (Fig. 5), although the effect of temperature was slightly below the 5% significance level for the strain 04-26 and at the 5% level of significance for the strain 04-28 (Table III). The effect of P concentration on trichome coiling parameters was not supported by RDA (Table III). Nevertheless, the intervals of 25% and 75% percentiles of coil diameters and distances in higher P concentrations ($WC_{0.1\times P}$, $WC_{1\times P}$, $WC_{10\times P}$) did not overlap those at lower P concentrations (Fig. 4). Together with increasing distances of adjacent trichome coils at low P concentrations ($WC_{0\times P}$, $WC_{0.001\times P}$), trichome coil diameters of the strain 04-28 decreased until almost straightened trichomes (Fig. 4). At higher P concentrations, the trichomes were coiled regularly, forming more or less tight spirals. After inoculation to fresh WC medium, the straightened trichomes from WC_{0xP} and $WC_{0.001\times P}$ media were able to grow into a culture of regularly coiled trichomes.

Temperature and light growth optima

Both the light and the temperature optima of the strains differed markedly and did not overlap in their ranges. The strain 04-26 displayed the temperature growth optimum between 17.5°C and 22.5°C and the light optimum 220–360 μmol m^{-2} s^{-1}, whereas the temperature growth optimum of the strain 04-28 was 22–28°C and the light optimum was 100–210 μmol m^{-2} s^{-1} (Fig. 6).

Secondary metabolite content

Secondary metabolite contents were determined in the strains studied. Only one compound was produced by both the strains 04-26 and 04-28. The identity of this compound (MW = 684, $m/z = 685$ $[M+H]^+$) is unknown.

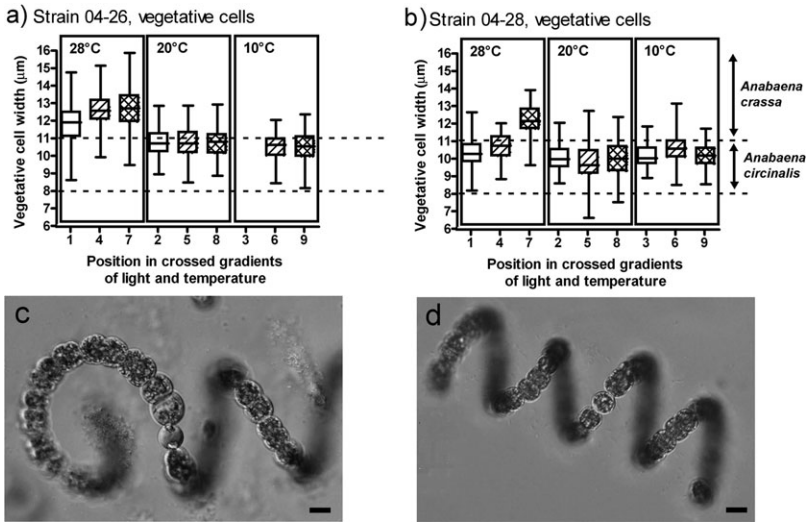

Fig. 2. Vegetative cell width of the strain 04-26 (**a, c, d**) and 04-28 (**b**) in various positions of crossed gradients of light and temperature. Light intensity is symbolized by shading (750 µmol m^{-2} s^{-1}, plain boxes; 200 µmol m^{-2} s^{-1}, simple shading; 20 µmol m^{-2} s^{-1}, double shading). Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated. Microphotographs show differences in vegetative cell width of the strain 04-26 grown at 28°C (**a**) and 10°C (**b**). Scale bars represent 10 µm.

All the other compounds identified in extracts of the strains 04-26 and 04-28 differed in their retention times and MS^2/MS^3 fragmentation spectra (data not shown).

Eight compounds were clearly identified from the mass spectra of strain 04-26. Molecular weights of these compounds were 845, 713, 887, 910, 755, 684, 812 and 1132. No ion corresponding to the loss of an amino acid was found, excluding the possibility that this compound was a peptide.

Five principal compounds were identified in the extract of strain 04-28. The molecular ions and related sodium and potassium adducts confirmed the presence of compounds with molecular weights 452, 684, 786, 870 and 854. None of these compounds were identified as a known structure. Analysis of the MS^2 spectra of molecular ions 786, 870 and 854 revealed one intensive peak corresponding to loss of water and/or CO_2. Further fragmentation of this ion led to the creation of fragments

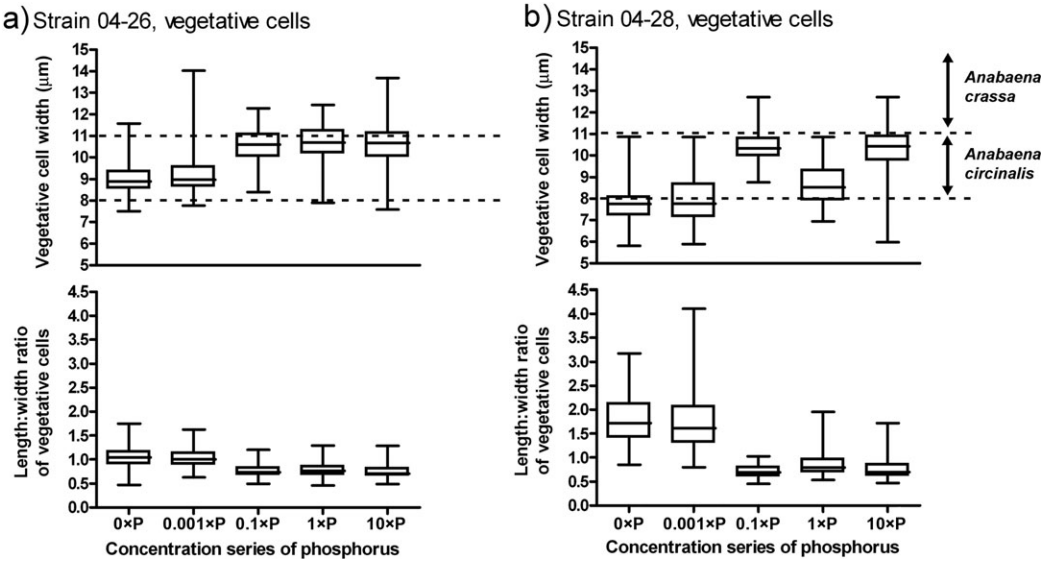

Fig. 3. Vegetative cell morphometric characteristics of the strain 04-26 (**a**) and 04-28 (**b**) under various phosphorus concentrations. Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated.

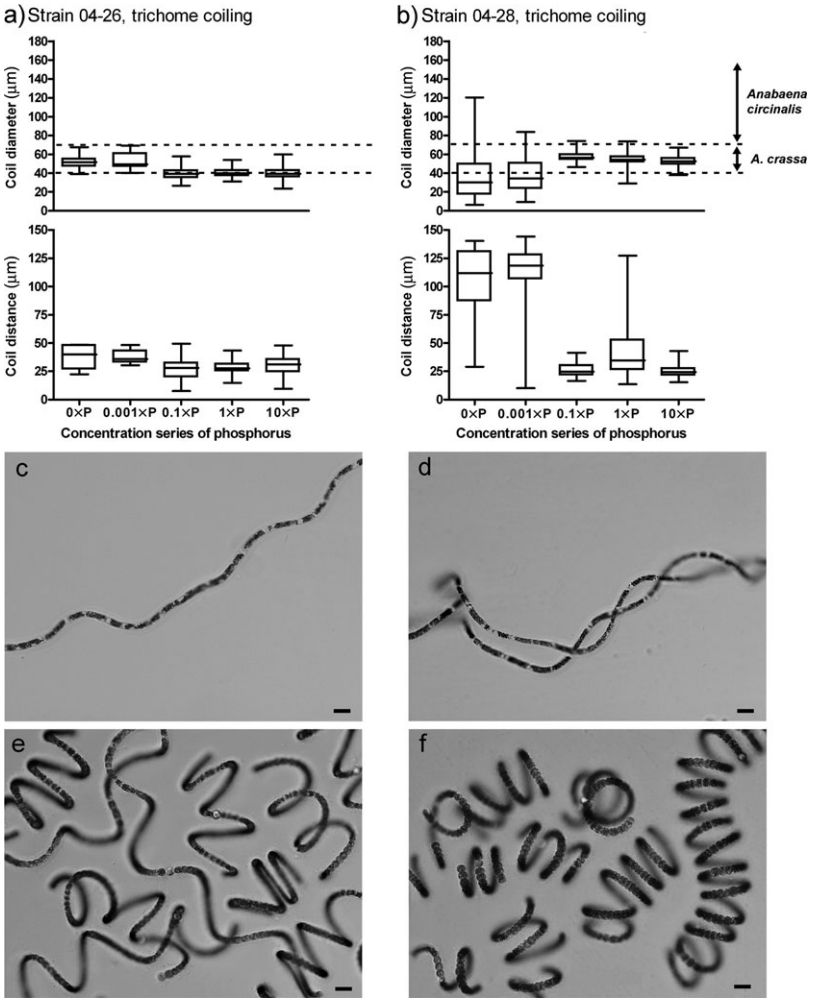

Fig. 4. Parameters of trichome coiling of the strain 04-26 (**a**) and 04-28 (**b**–**f**) under various phosphorus concentrations. Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated. Microphotographs demonstrate the variability in trichome coiling of the strain 04-28 under different phosphorus concentrations: (**c**) 0 µmol L^{-1} (WC_{0xP}); (d) 2.6×10^{-2} µmol L^{-1} ($WC_{0.001xP}$); (**e**) 2.6×10^{1} µmol L^{-1} (WC_{1xP}); (**f**) 2.6×10^{2} µmol L^{-1} (WC_{10xP}). Scale bars represent 20 µm.

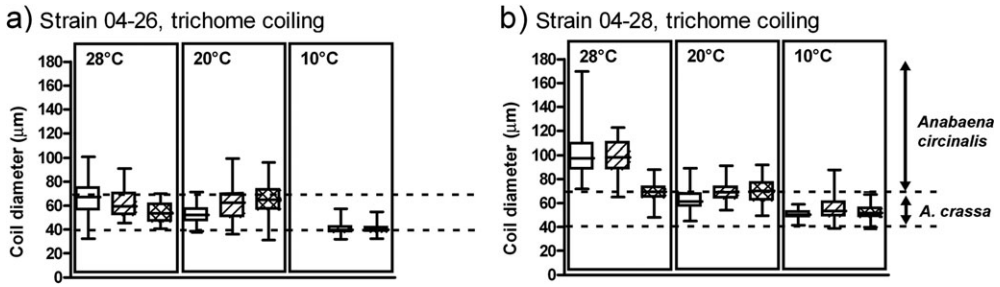

Fig. 5. Parameters of trichome coiling of the strain 04-26 (**a**) and 04-28 (**b**) in various positions of crossed gradients of light and temperature. Light intensity: 750 µmol m^{-2} s^{-1}, plain boxes; 200 µmol m^{-2} s^{-1}, simple shading; 20 µmol m^{-2} s^{-1}, double shading. Whiskers represent the minimal and maximal values, boxes symbolize the 25 and 75% percentiles and lines inside the boxes show the mean values. Ranges of values for the morphospecies *Anabaena circinalis* and *A. crassa* after Komárek (Komárek, 1996) are indicated.

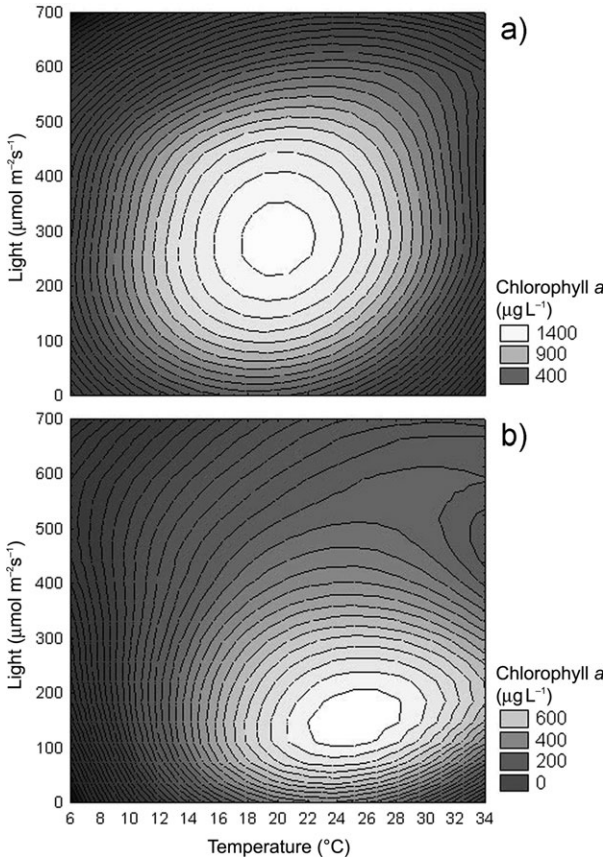

Fig. 6. Contour plots describing chlorophyll *a* concentrations ($\mu g\,L^{-1}$) of the strains as a function of temperature and light: (**a**) strain 04-26, (**b**) strain 04-28. Identical inocula of each strain were exposed to various combinations of temperature and light intensity. The biomass was harvested in exponential phase of growth of the fastest growing cultures.

that corresponded to amino acid loss. Fragmentation of the compound of MW 786 resulted in the loss of Gly, Ala, Val and Asn. Fragmentation of the compound of MW 870 resulted in the loss of Ala, Val, Lys/Gln and Met. Fragmentation of the compound of MW 854 resulted in the loss of Ala, Leu/Ile, Asp and Lys/Gln.

16s rRNA gene structure

Partial 16S rRNA sequences (1212 bp) of the strains 04-26 and 04-28 were compared with sequences of planktonic *Anabaena* strains available in GenBank. NJ and MP phylogenetic algorithms produced similar topologies, and therefore only the NJ tree is presented (Fig. 7). Both the strains exhibited 100% sequence identity and appeared in a common cluster together with *A. circinalis, A. crassa* and *A. planctonica* strains from lake Tuusulanjärvi, Finland (cluster A). Sequences of the 16S rRNA genes of all these strains were almost identical (similarities higher than 99.8%).

DISCUSSION

Morphology

Morphological plasticity under varied experimental conditions was described for the first time in the cyanobacterial species complex *Anabaena circinalis/A. crassa*. In most cases, the responses of morphological characteristics to varied conditions (temperature, light, nitrogen, phosphorus) were similar in both the strains, including *F*- and *P*-values of RDAs and percentages of the variability explained. This indicates that the results may be generalized to the whole group of similar planktonic *Anabaena* morphotypes, corresponding to the "large" species of Rajaniemi (Rajaniemi *et al.*, 2005b). Saker and Neilan (Saker and Neilan, 2001) found a similar strong consistency in morphological responses of seven *Cylindrospermopsis raciborskii* isolates to different nitrogen sources. The present study is the first report of significant relationships between *Anabaena* morphology and environmental parameters, since the only previous study dealing with effects of growth conditions (light and temperature) on *Anabaena* strains referred to the stability of *Anabaena* morphology (Stulp and Stam, 1985).

Our experiments demonstrated that both the strains studied covered the range of variability of both *A. circinalis* and *A. crassa*, as they were originally described. The strains displayed the range of trichome width of 8.5–13.5 µm (strain 04-26) and 7.0–13.0 µm (strain 04-28). The widest trichomes (12–13 µm) were observed at the highest experimental temperature (28°C), the thinnest trichomes (8–9 µm) at the lowest concentrations of phosphorus (WC_{0xB} $WC_{0.001xP}$). Diameters of trichome coils of strain 04-26 were 35–62 µm, whereas strain 04-28 had a wider range (15–110 µm). These results suggest that no reliable morphological criterion exists for distinguishing *A. circinalis* and *A. crassa*. This agrees with the suggestion made by Zapomělová *et al.* (Zapomělová *et al.*, 2007), who observed continuous variability of trichome widths and coil diameters of 13 *A. circinalis* and *A. crassa* populations in the Czech Republic. Consequently, a revision of these two *Anabaena* species is required, using a combination of molecular and morphological methods applied to different strains of these cyanobacteria.

An important finding of our study is the stability of akinete morphometry under varied experimental conditions. Thus, the dimensions and the shape of akinetes appear to be reliable criteria for identification of these *Anabaena* morphospecies, discriminating them from

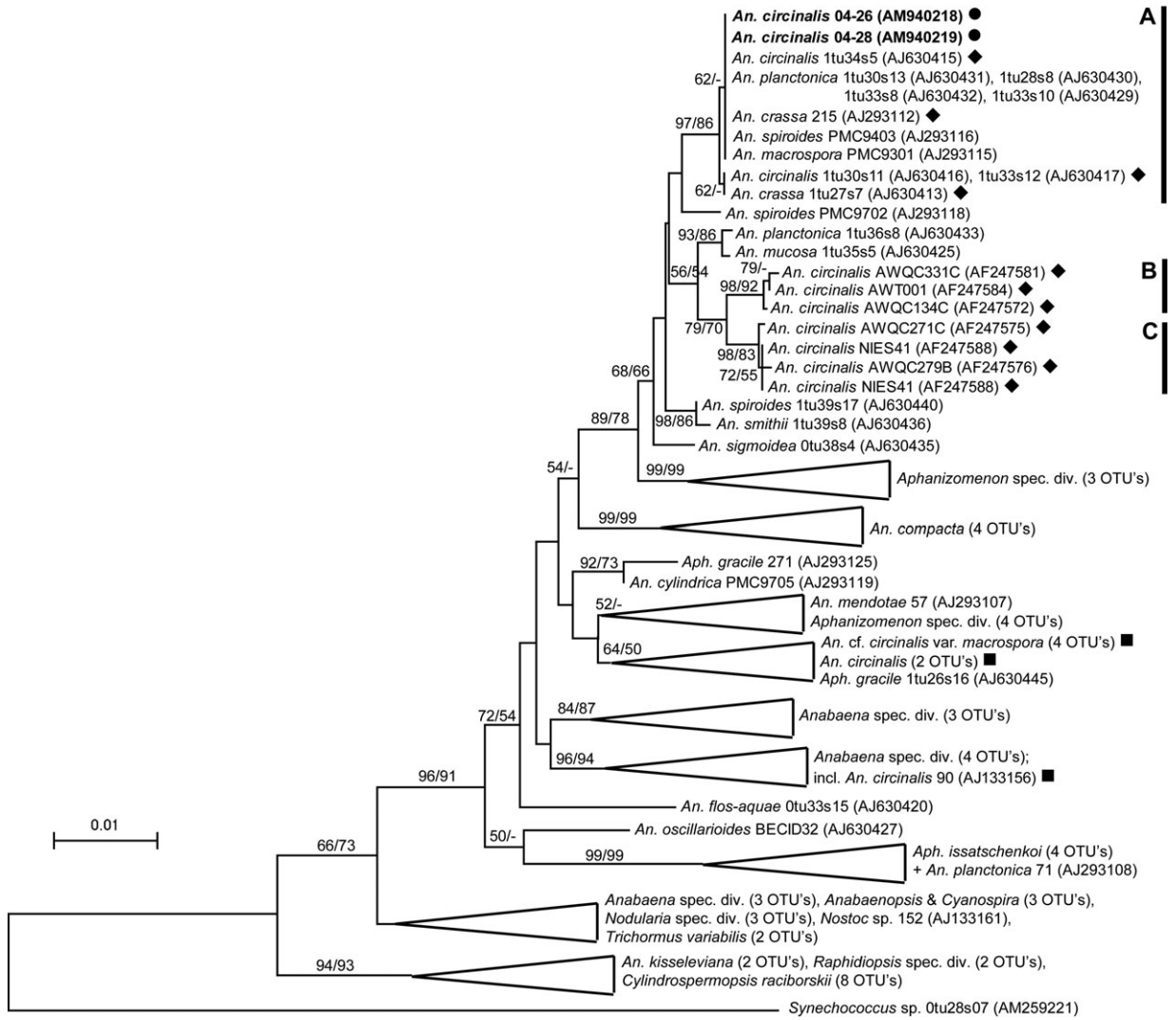

Fig. 7. NJ tree based on 16S rRNA gene sequences (1212 bp) showing the clustering of studied strains 04-26 and 04-28 (highlighted) with *A. circinalis* and *A. crassa* strains from Finland (cluster A). Numbers near the nodes indicate the bootstrap values over 50% for NJ and MP analyses. Abbreviations and symbols: *An*., *Anabaena*; *Aph*., *Aphanizomenon*; incl., including; OTU, operational taxonomic unit; spec. div., different species; filled circle, studied *Anabaena circinalis* strains; filled diamond, *A. circinalis* and *A. crassa* strains published by other authors; filled square, misidentifications of *A. circinalis* in other studies.

morphospecies with similar trichome width, such as *A. mucosa* Komárková et Eloranta 1992 and *A. ucrainica* (Schkorb.) M. Watanabe 1996.

Our study has demonstrated a high plasticity of trichome coil diameters and distances between adjacent coils under varied experimental conditions. An analogous conclusion has been derived from the study of Zapomělová *et al*. (Zapomělová *et al*., in press), who observed modifications in trichome coiling of *A. spiroides* related to the composition of the culture medium. Numerous reports on trichome straightening of *Anabaena* strains in cultures have been published (Booker and Walsby, 1979; Hickel, 1982; Zapomělová, 2004; Zapomělová *et al*., in press), indicating the instability of *Anabaena* trichome coiling in general. Recently published analyses of 16S rRNA, *rpoB* and *rbcLX* sequences have shown that trichome coiling does not reflect phylogeny, since *Anabaena* strains with coiled and straight trichomes, but with similar morphometric characteristics, often appear in the same clusters in phylogenetic trees (Beltran and Neilan, 2000; Rajaniemi *et al*., 2005a, b).

Temperature

Our experiments confirmed temperature as an important factor influencing growth and morphology of planktonic

Anabaena. A significant effect of temperature was found on morphometry of vegetative cells and heterocytes and on trichome coiling parameters. Previously published papers have emphasized the importance of persistent thermal stratification for the occurrence of blooms of *A. circinalis* (Mitrovic *et al.*, 2003; Westwood and Ganf, 2004a, b). It is controversial whether this is due to enhanced metabolic rates at higher temperatures (Robarts and Zohary, 1987) or more likely the result of the physical and chemical status of the water column (Mitrovic *et al.*, 2003). Accumulation of metabolic products as a result of enhanced metabolic rates may be a potential explanation of the vegetative cell and heterocyte enlargement that we observed in our strains at 28°C.

The temperature optima of our strains differed one from another but both of them corresponded more or less to the optimum of *A. circinalis* (20–25°C) reported by Tsujimura and Okubo (Tsujimura and Okubo, 2003). Inter-strain variability can probably be expected in temperature and light preferences, since Rapala and Sivonen (Rapala and Sivonen, 1998) referred to strain-specific differences in growth rates of various *Anabaena* strains as a function of temperature and light. On the contrary, Stulp and Stam (Stulp and Stam, 1985) demonstrated similar growth response to temperature and light of various representatives of one and the same morphological group of *Anabaena*.

The growth optimum of strain 04-28 was slightly higher than the water temperature commonly observed in Czech water bodies during the summer, which is 21–24°C (Znachor *et al.*, 2006). A similar finding was published from the Baltic Sea where the water temperature fluctuates around 15°C in summer, whereas the optimum growth temperature of a *Nodularia* strain isolated from these waters was 25–28°C (Lehtimäki *et al.*, 1997). One explanation for these discrepancies may be the adaptation to higher temperature during cultivation. This, however, seems unlikely in the case of our strain since both the strains studied were cultured at 21°C, which is lower than the temperature optimum determined for strain 04-28.

Light

A significant effect of light on morphological features of our strains was not observed, although physiological consequences (photoinhibition) could be expected from the high light intensities reached in the crossed gradients (Wyman and Fay, 1987). Similarly, the lowest light intensities of the crossed gradients can be considered limiting for cyanobacterial growth (Wyman and Fay, 1987). Our findings agree with the results of Stulp and Stam (Stulp and Stam, 1985) who also did not observe any effect of light on morphology of *Anabaena* strains.

A decrease in gas vesicle content was observed in our strains with rising light intensity and with decreasing P concentration, which is consistent with previous findings (Reynolds and Walsby, 1975; Konopka *et al.*, 1987; Brookes and Ganf, 2001).

The light optima of our strains were narrow, falling at the higher end of the range of light intensities determined optimal for the growth of *A. circinalis* (Westwood and Ganf, 2004b).

Nutrients (phosphorus, nitrogen)

Phosphorus concentration was the main factor influencing the morphology of vegetative cells, and consequently, trichome coiling characteristics were modified in the strains studied. Significant differences in the trichome width were previously observed between P-limited and non-limited populations of *C. raciborskii*. The thinner form from P-limited conditions was able to grow into normal-sized trichomes under favourable culture conditions (Komárková *et al.*, 1999), which agrees with our results.

The phosphorus-dependent trichome straightening of the strain 04-28, which was observed in our experiments, is not consistent with the ecological context. Trichomes should tend to sink to lower parts of the water column when P is limited in the euphotic zone, whereas trichome straightening would counteract this (Booker and Walsby, 1979; Padisák *et al.*, 2003). It seems to be more likely a geometrical consequence rather than an ecological adaptation. The cells of the strain 04-28 became longer at the lowest P concentrations, supposedly because of the lack of sources necessary for cell division. Then the increased cell length affected the trichome shape.

The insignificant effect of low nitrogen concentrations can be easily explained by the N_2-fixing ability of *Anabaena* (heterocytes). However, our study also demonstrated that the high N concentrations achievable in field had no effect on *Anabaena* morphology. This contrasts with the results of Saker and Neilan (Saker and Neilan, 2001) who found significant response of morphological variability of *C. raciborskii* strains to different sources and concentrations of N.

Secondary metabolite content

Despite high morphological and 16S rRNA gene sequence similarities, we found a very low similarity in the production of secondary metabolites between the two strains studied. This finding is consistent with the

published results on discrepancies between phylogenetic features and protein or toxin production (Palinska *et al.*, 1996; Bolch *et al.*, 1999; Iteman *et al.*, 2002). The random distribution of peptide synthetase genes across the phylogentic spectrum was demonstrated by Rantala *et al.* (Rantala *et al.*, 2004) in the case of microcystin synthesis. The authors suggested that the ability to synthesize different peptides is scattered across the phylogenetic spectrum because of extinction of the ancient synthetic pathway in different evolution lineages. We have shown that even cyanobacteria within one lineage can produce markedly different types of metabolites. This can refer to fast changes in regulation and pattern of non-ribosomal synthetic pathways, the pathways which cyanobacterial peptides are synthesized by (Welker and von Döhren, 2006). Secondary metabolite production can vary considerably in response to growth conditions (Rapala *et al.*, 1993, 1997) or to the growth phase (Negri *et al.*, 1997). However, the effect of the above-mentioned factors seems to be limited in this case, since both of our strains were isolated almost at the same time, kept under identical culture conditions and harvested in exponential phase of growth.

Phylogenetic relationships based on 16s rRNA gene structure

Our strains displayed very high 16S rDNA sequence similarities with all Finnish *A. circinalis* and *A. crassa* strains (marked with diamonds in Fig. 7) corresponding to subcluster A of Rajaniemi *et al.* (Rajaniemi *et al.*, 2005a, b). On the other hand, Australian strains of *A. circinalis* (Beltran and Neilan, 2000), which were located separately from the European cluster, were divided into two distinct branches of higher heterogeneity (clusters B and C). The strains designated as *A. circinalis* var. *macrospora* by Rajaniemi *et al.* (Rajaniemi *et al.*, 2005a, b) or as *A. circinalis* in other studies (Lyra *et al.*, 2001; Gugger *et al.*, 2002) were placed into clusters considerably distant from all other *A. circinalis* strains in the present phylogenetic tree (marked with squares in Fig. 7). Morphology of the strains of *A. circinalis* var. *macrospora* evidently differed from the "true" *A. circinalis* strains described in the same paper (Rajaniemi *et al.*, 2005a) and also from our strains 04-26 and 04-28. Phenotypic characteristics of the strains *A. circinalis* studied by Lyra *et al.* (Lyra *et al.*, 2001) and Gugger *et al.* (Gugger *et al.*, 2002) were not published in detail but presumably, these strains belonged to *Anabaena* morphotypes with thinner trichomes since they appeared near *A. circinalis* var. *macrospora* or even separately.

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