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MORPHOLOGICAL CHARACTERISTICS OF *CYLINDROSPERMOPSIS RACIBORSKII* (WOŁOSZYŃSKA) SEENAYYA ET SUBBA RAJU IN LABORATORY CULTURES

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The freshwater cyanoprokaryote *Cylindrospermopsis raciborskii* has become increasingly prevalent in tropical and temperate water bodies worldwide. The morphological characteristics of this species were investigated under different growth rates in continuous cultures (at steady state) and in batch (phosphorus starved) cultures with different mineral nitrogen forms. The species displays an enormous morphological variability under controlled condition. The occurrence of extreme long twisted filaments was found near the maximum growth rate and under high ammonium concentration. Rarely the heterocytes of *Cylindrospermopsis raciborskii* arise intercalarly between two neighbouring cells (i.e. intercalary heterocytes were found). The morphological features are highly effected by environmental conditions and nutrient availability. Under P-starvation extreme morphology appeared. The specifications of *C. africana* and *C. cuspis* overlap with that of *C. raciborskii* accordingly this is not clear characteristic feature to distinguish species. A pure culture of a pro- or eukaryote alga growing in continuous cultures is a good method for giving a suitable overview on all morphological possibilities of a tested organism.

Keywords: Batch and continuos cultures – *Cylindrospermopsis raciborskii* – morphology – P-starvation – nitrogen forms

INTRODUCTION

Seenayya and Subba Raju [24] transferred *Anabaena (Anabaenopsis) raciborskii* (straight trichome) [31] into a new monotypic genus *Cylindrospermopsis* according to the development of heterocytes (heterocysts). In *Cylindrospermopsis*, heterocytes develop primarily by the terminal cells (on one end or on both ends of a trichome). The terminal cell divides into two equal parts and then a heterocyte is formed from the apical daughter one, and the subapical cell grows up and can divide again as other vegetative cell [5]. In genus *Anabaenopsis* heterocytes are formed in the intercalary position in pairs by conspicuously unequal division of two neighbouring cells. Jeeji Bai et al. [10] considered that the creation of the genus *Cylindrospermopsis* had been unjustified and delimitation from *Cylindrospermum* was made only by the position

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of akinetes which is not always in the intercalary position in *A. raciborskii*. Many authors [5, 7, 20] agreed unanimously on that *Cylindrospermopsis* differs from *Cylindrospermum* in respect to: (i) the presence of gas vacuoles that are absent in *Cylindrospermum;* (ii) the heterocyte-free end of trichomes have attenuated and pointed ends; (iii) akinetes (when formed) are separate from the terminal heterocyte by 1 or 2–3 vegetative cells, while in *Cylindrospermum* heterocysts are generally jointed with one or two akinetes, i.e. there are no vegetative cells between the heterocytes and the akinetes.

Other species of *Cylindrospermopsis* have also been described [12, 13, 14, 29]. Some extreme forms of the morphology of *Cylindrospermopsis raciborskii* were recorded in northern Australia [22], and in Brazil [15].

The most frequently reported species of genus *Cylindrospermopsis* is *C. raciborskii,* which is able to produce toxic product [2, 23] such as other *Cylindrospermopsis*-like species. Therefore, the morphology of this genus is important for the correct identification. It has special importance from ecological and toxicological point of views. However, it is impossible to identify the different *Cylindrospermopsis* species in natural sample by DNA analysis.

There is little morphological information based upon the cultured *Cylindrospermopsis* under well-defined conditions. The continuous culture technique is advantageous in the studies of the changes of morphological and biochemical activities of a pure isolated alga by changing growth rate, physical or chemical conditions. This agrees with the requirement of the modern taxonomic system of cyanobacteria, which is now generally accepted [18]. This study investigates the effects of well-defined growth conditions in batch and continuous cultures on morphology features and the dimensions of vegetative-cells, trichomes and heterocytes to detect the possibility of morphological variance of *Cylindrospermopsis raciborskii*. The results were discussed with some natural samples.

MATERIALS AND METHODS

The ACT 9502 strain of *C. raciborkii* was isolated from Lake Balaton by A. Kovács. The strain is maintained in a modified BG-11 medium at a temperature of 22 + 1 °C and 140 μmol m^{-2} s^{-1} (see the details [9, 25, 28]).

Culture conditions

Batch and continuous cultures (Table 1) were run under the same light and temperature. Continuous light with irradiant of 120 μMol m^{-2} s^{-1} and temperature of 24 ± 1 °C were used. The culturing medium was the same as that used by Shafik et al. [26], except the concentrations of phosphorous and nitrogen (see below).

Batch cultures (P-starved cultures)

In the batch cultures experiments three chemostat vessels of 2 liters capacity were used. In each vessel 25 ml of the stock culture of *C. raciborkii* was inoculated into two liters of P-free medium. In the first vessel the culture medium was reached by 300 µg NH_4-N l^{-1}, in the second by 300 µg NO_3-N l^{-1} and in the third one the atmospheric nitrogen was the nitrogen source. By day 2 the concentration of SRP was undetected in all cultures. Samples were harvested after thee weeks and the changes in morphology were investigated.

Chemostat cultures

The chemostat apparatus that used in these experiments have been described in details by Shafik et al. [26]. In these cultures 5 mg PO_4-P l^{-1} was used in the inflowing medium. Three groups of experiments (Table 1) were run under five dilution rates of 0.15, 0.25, 0.50, 0.75 and 0.93 d^{-1} (near the maximum growth rate) with two nitrogen sources (NO_3 or NH_4) as follows.

Table 1
The growth conditions used through the study

Culture system	Growth rate (d^{-1})	Nitrogen source	Nitrogen concentration in the inflowing medium (µg/l)	Mode of nitrogen supply
Batch P-starved	–	N_2, NO_3 or NH_4	–	–
Chemostat	0.150	NH_4	300	Continuous
Chemostat	0.250	NH_4	300	Continuous
Chemostat	0.500	NH_4	300	Continuous
Chemostat	0.750	NH_4	300	Continuous
Chemostat	0.930	NH_4	300	Continuous
Chemostat	0.150	NH_4	300	Pulsed
Chemostat	0.250	NH_4	300	Pulsed
Chemostat	0.500	NH_4	300	Pulsed
Chemostat	0.750	NH_4	300	Pulsed
Chemostat	0.930	NH_4	300	Pulsed
Chemostat	0.150	NO_3	300	Continuous
Chemostat	0.250	NO_3	300	Continuous
Chemostat	0.500	NO_3	300	Continuous
Chemostat	0.750	NO_3	300	Continuous
Chemostat	0.930	NO_3	300	Continuous
Chemostat	0.500	NH_4	750	Continuous
Chemostat	0.500	NH_4	1500	Continuous
Chemostat	0.500	NH_4	3000	Continuous

1. Chemostat cultures with continuous supply of 300 μg NH_4-N l^{-1} (NCR); 2. Chemostat cultures with pulsed supply of 300 μg NH_4-N l^{-1} (NPR) and 3. Chemostat cultures with continuous supply of 300 μg NO_3-N l^{-1} (NOR).

Three more continuous cultures were run with NH_4-N concentrations of 750, 1500 and 3000 μg l^{-1} at a dilution rate of 0.5 d^{-1}. After the cultures had reached a steady state [dilution rate (D) = growth rate (μ)] a definite volume of 20 ml samples were preserved by Lugol's solution and stored in an ice-box (4 °C) until examination.

A camera (RT color, Diagnostic instruments. inc. SPOT, USA, with computer program) coupled with a microscope (Nikon, Japan) was used for the examination of morphological features diagnostic and for detection of the cell, trichome and heterocyte dimensions and volume of this cyanoprokaryote. A minimum number of 30 trichomes per sample were examined and measured in detail. The volume of the cells and filaments were calculated from the length and the diameter measurements as cylinder-shape.

RESULTS

Morphology and dimensions in batch cultures

In batch (P-starved) cultures cells were short, straight or slightly curved, fragile. Terminal cells had bluntly or sharply pointed ends (Fig. 1). The length of the terminal cells was 5.1 ± 0.9 μm. The cell dimensions were from 3.96 to 7.63 μm length and from 0.76 to 1.34 μm wide with L/W ratio between 4.67 and 6.93. The trichomes were straight and short with a length of 7.55–68.68 (76.8) μm and the width of trichomes [0.76–1.04 (1.37) μm] was much thinner than in the chemostat cultures, some trichomes had irregular morphology and consisted of one, two or four cells (Fig. 1). The width and length of heterocytes were much smaller in the P-starved cultures than in the chemostat cultures (see below). The width was 1.47 ± 0.4 μm and the length was 5.37 ± 0.88 μm (n = 28). The heterocytes of batch cultures have special morphological characteristics different from that of chemostat cultures; they have a conical or drop-shape (Fig. 2). The number of heterocytes per filament was 1.75 ± 0.5 in the batch (P-starved) cultures. Many free heterocytes have been observed in these cultures i.e. under this condition the filaments easily lose the heterocyte(s).

Morphology and dimensions at different growth rates in continuous cultures

Morphology and dimensions of cells

Cells are cylindrical, sometimes elongate barrel shaped in all cultures. Cell length was only measured for the cell that has clearly observed cross-wall in trichomes. Sometimes the cross-wall was unclear through the trichome. The cell length and

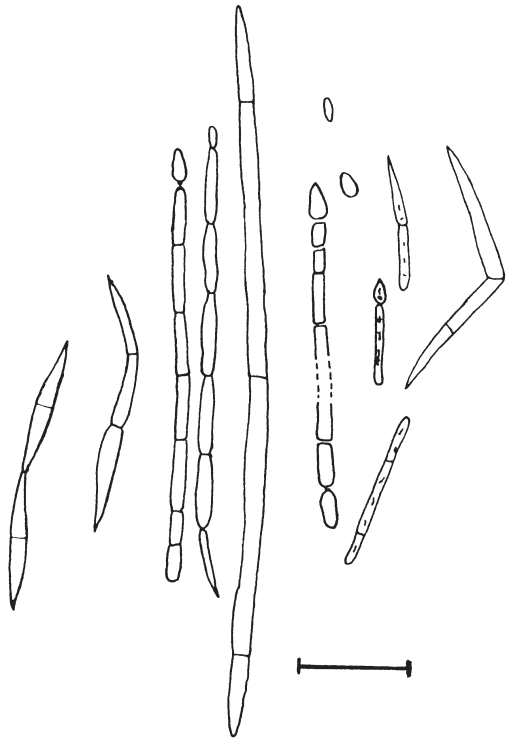

Fig. 1. Morphology of cells, trichomes and heterocytes in batch (P-starved) cultures. Bars = 10 μm

widths varied with the growth rate, the nitrogen source and the regime of N supply (Table 2). The maximum length and width were recorded at a growth rate of 0.5 d^{-1} (near the half maximum growth rate) in ammonium pulsed cultures. The pulsed regime supported the largest cells of 139 ± 5.5 μm^3 [19.41 (8.83) × 3.02 (0.90) μm] at growth rate of 0.5 d^{-1} (Fig. 2A, Table 2). One-way ANOVA on the cell length and cell width indicated significant correlation of the growth rate ($p \bullet \bullet 0.0001$ in all regimes; F = 131.15 for width and 75.85 for length, n = 178 in NCR; F = 116.92 for width and 61.92 for length, n = 184 in NPR; F = 201.14 for length, 197.72 for width, n = 195 for NOR). More often than not near the ends of the filament the lengths of cells were relatively shorter. The length of the terminal cells was 11.3 ± 2.0 μm in all chemostat cultures.

Morphology and dimensions of trichomes

The trichomes were straight or slightly curved and the average length of trichomes was 136.4 ± 46.1 μm in all cultures. Except when the cyanoprokaryote was grown

Table 2
Main dimensions of cells, trichomes and heterocytes of *C. raciborskii* at 300 µg N l^{-1} in different growth rates (see Table 1)

D (d^{-1})	NH_4 pulsed (NPR)			NH_4 continuous (NCR)			NO_3 (NOR)		
	Length (µm)	Width (µm)	L/W ratio	Length (µm)	Width (µm)	L/W ratio	Length (µm)	Width (µm)	L/W ratio
					Cells				
0.15	9.46(2.61)	2.50(0.69)	3.78	12.84(5.03)	2.77(0.45)	4.64	10.84(5.73)	2.72(0.61)	3.99
0.25	18.87(7.67)	2.84(0.55)	6.64	9.71(2.21)	3.00	3.24	14.17(7.07)	2.71(0.82)	5.22
0.50	19.41(8.83)	3.02(0.90)	6.43	16.58(5.28)	2.46(0.54)	6.74	14.00(2.84)	2.57(0.51)	5.45
0.75	14.52(3.94)	2.60(0.38)	5.59	11.44(4.51)	2.35(0.78)	4.88	16.63(4.62)	2.60(0.68)	6.41
0.93	13.26(4.95)	2.27(0.27)	5.85	9.35(2.88)	2.71(0.36)	3.45	13.37(4.21)	2.83(1.04)	4.72
					Trichomes				
0.15	105.30(41.60)	2.50(0.69)	42.05	216.75(88.49)	2.77(0.45)	78.34	149.98(82.10)	2.72(0.61)	55.19
0.25	209.26(121.54)	2.84(0.55)	73.62	232.00(0.00)	3.00(0.00)	77.33	302.98(221.02)	2.71(0.82)	111.63
0.50	227.05(115.56)	3.02(0.90)	75.19	169.92(86.51)	2.46(0.54)	69.05	145.54(53.72)	2.57(0.51)	56.65
0.75	100.79(32.07)	2.60(0.38)	38.80	107.48(41.62)	2.35(0.78)	45.81	153.35(57.15)	2.60(0.68)	59.09
0.93	107.93(26.99)	2.27(0.27)	47.63	121.00(36.22)	2.71(0.36)	44.70	1336.58(560.07)	2.83(1.04)	471.95
					Heterocytes				
0.15	7.65(1.02)	2.28(0.62)	3.35	8.42(1.74)	2.64(0.70)	3.19	7.74(2.17)	2.64(0.75)	2.93
0.25	11.13(3.24)	3.40(0.68)	3.27	8.50	3.00	2.83	9.51(2.35)	3.12(0.90)	3.05
0.50	10.19(2.31)	2.67(0.66)	3.82	9.69(1.09)	2.48(0.43)	3.91	9.28(1.58)	2.52(0.42)	3.69
0.75	9.12(2.49)	2.08(0.35)	4.40	8.39(3.28)	2.00(0.68)	4.20	9.11(2.08)	2.24(0.42)	4.07
0.93	9.03(1.62)	2.41(0.55)	3.75	7.88(1.71)	2.81(0.70)	2.80	7.96(2.19)	2.27(0.30)	3.51

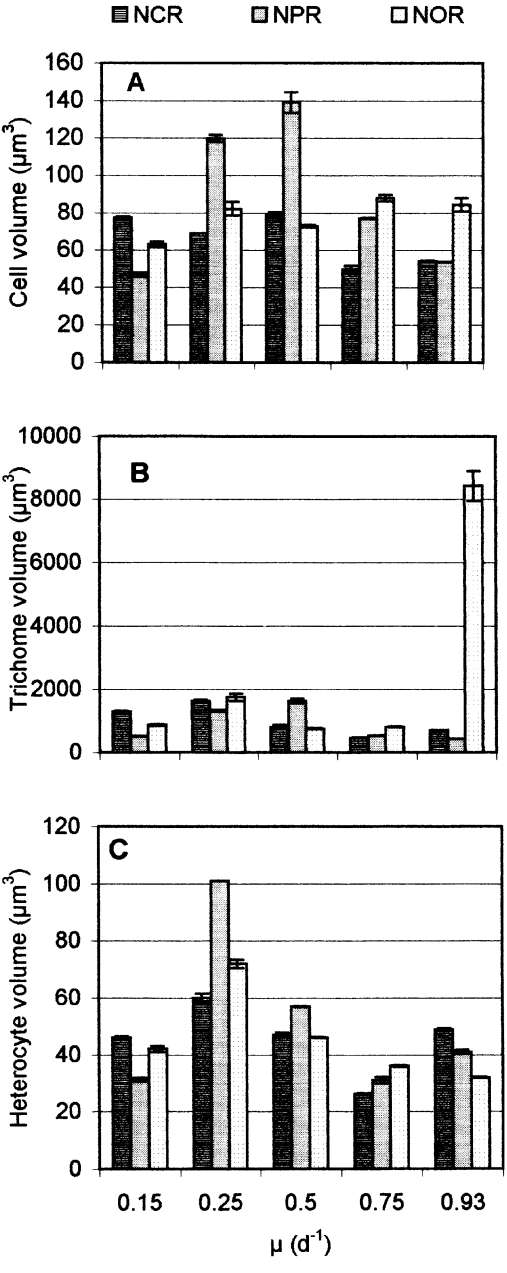

Fig. 2. Changes of volume of cells, trichomes and heterocytes of *C. raciborskii* at varies growth rates (µ, d^{-1}) under different nitrogen regimes, continuous ammonium regime (NCR), pulsed ammonium regime (NPR) and continuous nitrate regime (NOR). (A) cell volume; (B) trichome volume and; (C) heterocyte volume. The result is the main value of measured dimensions ±SD

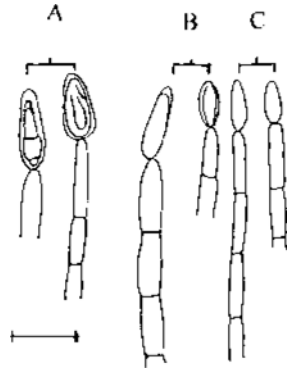

Fig. 3. Different forms of heterocytes and trichomes formed by *C. raciborskii* under different growth conditions in continuous cultures. (A) large heterocytes (rarely formed); (B) in pulsed regime; (C) in continuous regime. Bars = 10 μm

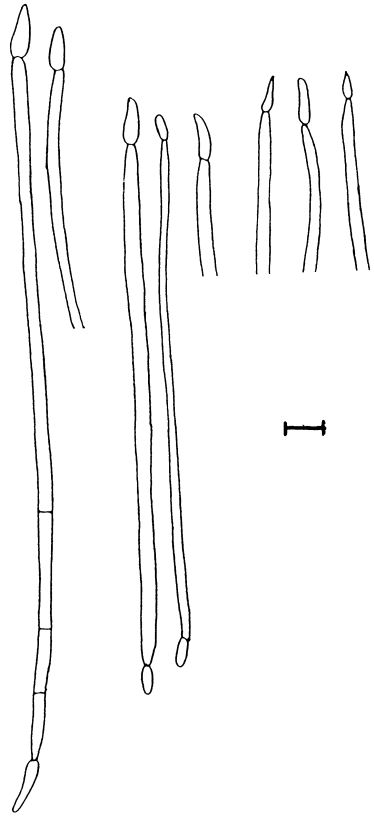

Fig 4. The morphological features of trichomes and heterocytes in continuous cultures show different shapes and size of heterocytes formed on the same trichome at the same time. Bars = 10 μm

near its maximum growth rate in NO_3-N culture, the trichomes were very long with an average length of 1.34 ± 0.6 mm and the main width of trichomes was 2.83 ± 1.04 μm (Table 2). Accordingly, the maximum trichome volume of 8422 ± 473 μm^3 was recorded at growth rate of 0.93 d^{-1} (Fig. 2B). At this growth rate all trichomes were twisted or irregularly bent. One-way ANOVA on the trichomes length on growth rate of chemostat cultures indicated significant effect for NCR ($F = 46.2$, $p \bullet \bullet 0.0001$, $n = 155$) and for NPR ($F = 28.6$, $P = 0.0007$, $n = 134$) but it was insignificant for NOR ($F = 3.2$, $P = 0.1$, $n = 182$).

Morphology and dimensions of heterocytes

Figures 3 and 4 show the different forms of heterocytes. The width of heterocytes was measured at the broadest point of each heterocyte. Table 2 shows the measurements of heterocytes under different regimes. The highest measurements of the length, the width [Fig. 5 (above)] and the volume of heterocyte were recorded at growth rates of 0.25 d^{-1} (Table 2, Fig. 2C). The pulsed regime supported larger heterocytes (101 μm^3) than the continuous regimes (60 ± 1.5 μm^3), at growth rates of 0.25 (Figs 2C, 3B, C). One-way ANOVA on the length and width of heterocytes indicated significant correlation of the dilution rate ($p < 0.0001$ in all regimes; $F = 84.48$ for width and 505.6 for length, $n = 102$ for NCR; $F = 116.92$ for width and 87.87 for length, $n = 98$ for NPR; $F = 215.4$ for length, 439.2 for width, $n = 95$ for NOR). There is a significant correlation between heterocytes volume and the growth rate ($p < 0.0001$) for all cultures.

The number of heterocytes per filament increased with the growth rate NPR and NOR. The average heterocytes number was much lower in pulsed regime (NPR) than in continuous regimes (NCR and NOR) [Fig. 5 (below)]. There is no significant correlation between the numbers of heterocytes per filament and the growth rate ($p > 0.02$) in all regimes.

Sometimes extreme shapes with great volume heterocytes were formed in the cultures (Fig. 3A). Rarely *C. raciborskii* forms intercalary heterocytes with the terminal heterocytes on the same filament (Fig. 6). It is not clear why these forms formed. Sometimes different morphological forms of heterocytes were found in the same culture (Fig. 4). No akinetes were appeared in any of the cultures.

Morphology and dimensions under different NH_4 concentrations in continuous cultures

The morphology and the dimensions of cells and filaments at different NH_4 concentrations, except at 3 mg l^{-1}, were in the usual dimensions. The average filament length was 127.3 ± 30.9 μm and width was 2.51 ± 0.06 μm (Fig. 7). The cell dimensions were 7.24 – 16.5 (23.1) × 1.53 – 2.51 (4.5) μm. The heterocyte dimensions were 3.77 – 7.8 (12.6) × 1.3 – 2.35 (3.4) μm. At the highest NH_4 concentration (3000

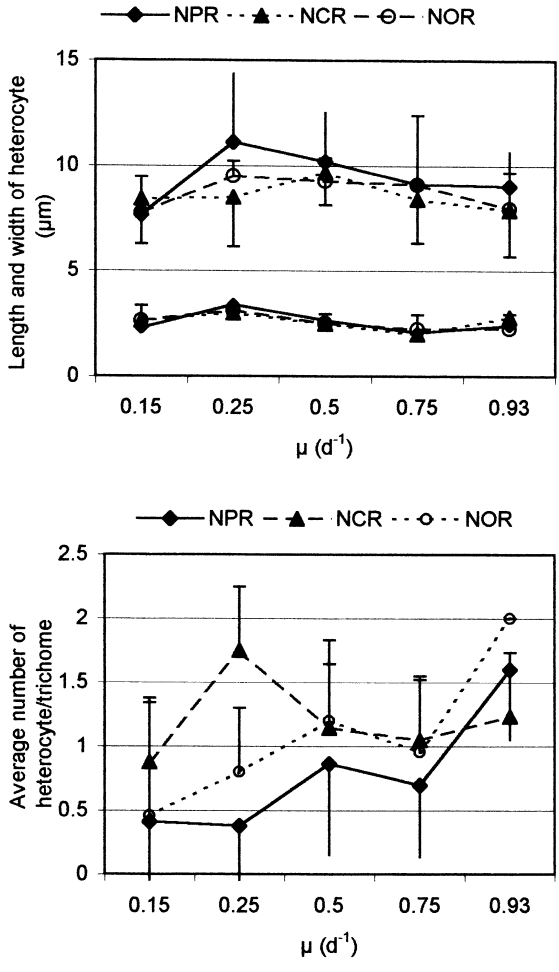

Fig. 5. Changes of length, width (above) and number of heterocytes per trichome (below) of *C. raciborskii* with growth rate (µ, d^{-1}) under different nitrogen regimes. Pulsed ammonium regime (NPR), Continuous ammonium regime (NCR) and Continuous Nitrate regime (NOR). The result is the mean value of measurements ±SD

µg l^{-1}) the trichomes were extremely long with a maximum length of 3.3 mm and all trichomes were twisted or irregularly bent as that formed at the maximum growth rate in NOR (Fig. 8). At lower concentrations the length and width of heterocytes were smaller. The average number of heterocytes per filaments decreased with the increase of NH_4 concentration. Figure 7 shows the effect of NH_4 concentration on the cell, filament and heterocyte measurements and the average numbers of heterocytes per filament. One-way ANOVA gives a significant correlation between the cell and filament measurements and NH_4 concentration where $p^{**} 0.0001$ for all cultures and

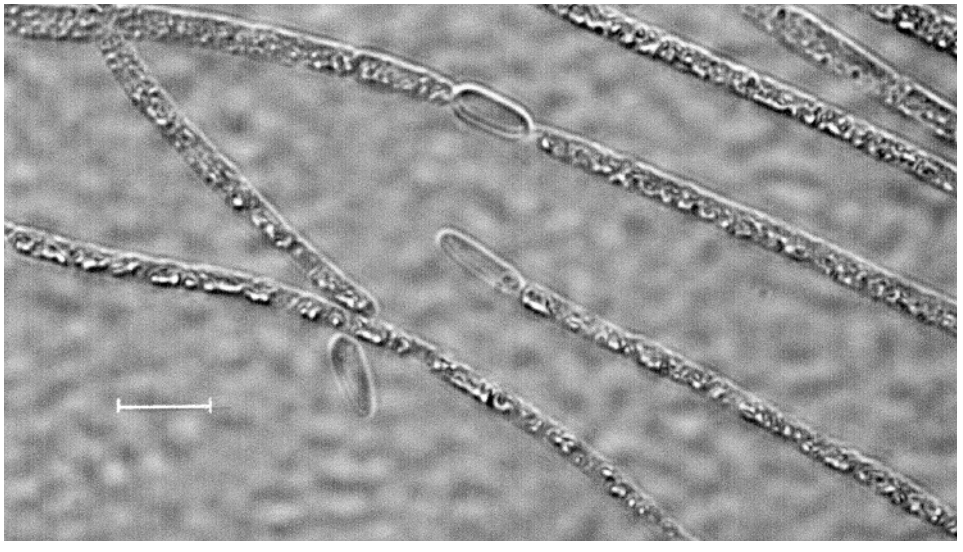

Fig. 6. Trichomes of *C. raciborskii* formed an intercalary and terminal heterocytes (B)

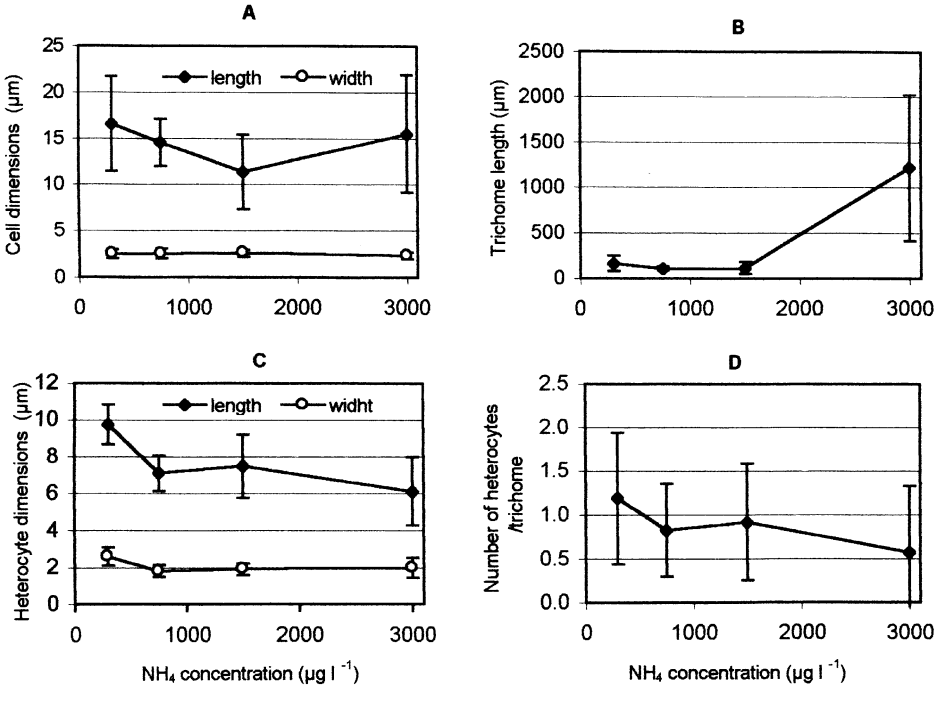

Fig. 7. Different measurements of vegetative cell (A), trichome (B), and heterocyte (C), and number of heterocyte per trichome (D) under various ammonium concentrations (see Table1). The result is the mean value of measurements ±SD

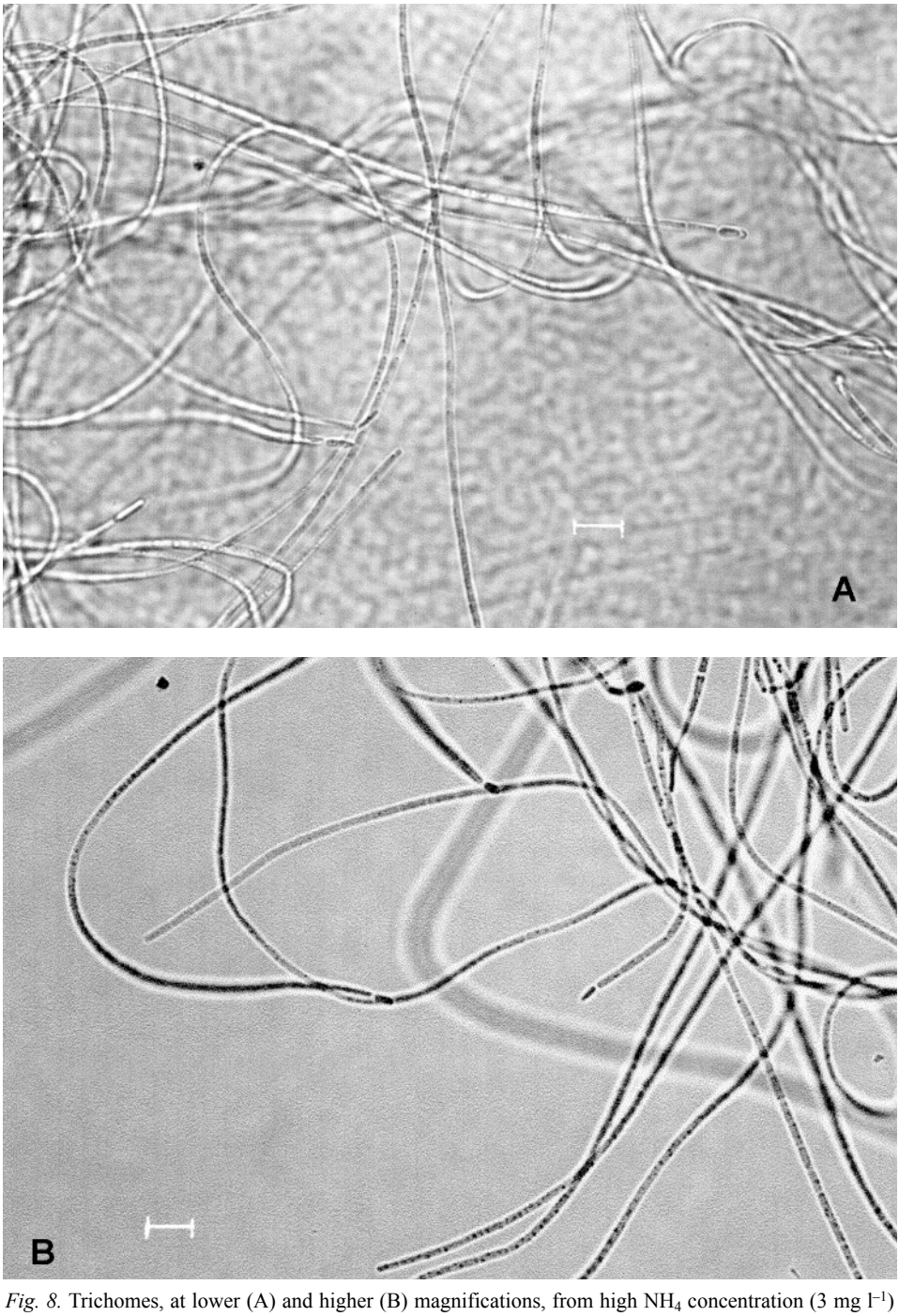

Fig. 8. Trichomes, at lower (A) and higher (B) magnifications, from high NH_4 concentration (3 mg l^{-1}) culture at growth rate of 0.5 d^{-1} show the long twisted form of filaments. Bars = 10 μm

F = 34.25 (n = 63) for trichome length, 84.3 (n = 96) for cell length and 94.3 (n = 159) for cell or trichome width. A significant correlation, except in the case of trichome volume (p = 0.11 and F = 2.47), was indicated between NH_4 concentration and all measurements.

DISCUSSION

Cylindrospermopsis is described as planktonic, trichomes solitary, straight, slightly curved or spirally coiled, sub-symmetric structure; gas vacuole is present; mucilaginous is envelope absent. Heterocytes are conical or long ovoid in shape, always terminal at one or both ends of the trichome. The ends of the trichome attenuated if heterocytes are absent; vegetative cells are cylindrical with little or no constriction at the cross-walls; akinetes are cylindrical or oblong-ovate, solitary or in pairs, intercalary, generally near to the end of trichome but remote from the heterocytes with para-heterocytic development.

The practical problem in identifying the species lies in the fact that the morphology of the trichome varies during population growth and under some conditions the akinetes and heterocytes are absent [20, 23, 30].

In continuous cultures growth rate, type of medium and regime of nutrient supply are the keys of control. The length and volume of cells and filaments that grew in P-starved cultures and sometimes at the lowest growth rate ($\mu = 0.15\ d^{-1}$) were smaller than that found in higher growth rates. If a nutrient element can be found in a low concentration or absents (e.g. under starved conditions or at very low growth rates) the cell quote is very low and dissolved nutrients must pass though the semi-permeable membrane into the cell. Thus, cells have to remain small with a favourable surface: volume ratio enabling efficient nutrient uptake [21]. These factors are effecting not only the cell and filament dimensions but also the morphological characteristics of *C. raciborskii.*

Comparing the extreme morphology of *C. raciborskii* reported by Komárková et al. [15] and P concentration in Lagoa do Peri lagoon in Brazil to the morphology of *C. raciborskii* in P-starved indicating that these extreme forms formed under P-starvation or limited condition. Lake Balaton, the source of the investigated stain, is a P-limited water body [8]. The P concentration in the lake water is less than 5 $\mu g\ l^{-1}$. Some extreme forms of *C. raciborskii* were recorded in the lake last year (Padisák, personal communication). Therefore, these forms may be indicating P-deficiency in nature.

Although the changes of trichome morphology during population growth were noted and described in 1939 [27] many more observations of morphology were recorded [1, 2, 3, 5, 6, 12, 13, 15, 23]. Flexible trichomes of more than 3 mm length with irregularly carved and/or bent wavy (Fig. 8) has not been recorded in field samples. This form and length are formed only near the maximum growth rate or high NH_4 concentration supply indicating that in nature this cyanoprokaryote rarely grow near its maximum growth rate. The spirally coiled trichomes characterized *C. philip-*

pinensis have never been observed in any cultures. Baker [2] discussed the simultaneous occurrence of coiled and straight forms, which were proved to be genetically identical according to 16s rRNA analysis and phycocyanin gene sequences. Generally, cell size and shape might not be under tight genetic control [4, 22]. In conclusion, again, the identification of *Cylindrospermopsis* cannot depend on the morphology and/or the length of trichome, especially if the heterocytes and akinates are absent. Komárkek and Kling [12] was classified *Cylindrospermopsis* form Lake Victoria (Africa) and over tropical regions into *C. raciborskii, C. africana* and *C. cuspis* and they professed that the variation in different of these species must be studied further under identical conditions. This classification may be incorrect for these reasons 1 the widths of cell or filament of *C. africana* and *C. cuspis* were observed in P-starved cultures, where only thinner trichomes in range of 0.76 – 1.37 μm width were formed. This range is closed to that of *C. cuspis*. 2. the terminal cells of the thinner trichomes have conical pointed or rounded ends at lower growth rate. Accordingly this character cannot differentiate *C. africana* and *C. cuspis*. 3. the cross-wall constrictions were clearer at higher NH_4 concentration, maximum growth rate and P-starved cell but it was a bit noticeable in other growth rates.

The development of the heterocytes and their frequency are dependent on the environmental conditions, particularly on the nitrogen uptake and nitrogen and carbon metabolisms [11]. With the exception of the lowest growth rate (0.15 d^{-1}) the volume of heterocyte decreased with the increasing of the growth rate while the number of heterocyte/trichome increased. The increase of live heterocytes with growth rate is recorded for *Anabaene flos-aquae* as a result of the increase of the heterocytes population size but there was no trend in heterocyte viability with growth rate [17]. For *C. raciborskii* there was no trend in number of heterocytes per filament and growth rate. Ammonium completely represses heterocytes formation in *Anabaene flos-aquae* cultures but in the case of *C. raciborskii* heterocytes are formed in all growth rates and at high NO_3 [26] or high NH_4 concentration (manuscript in preparation). The volume and the number of heterocytes decreased with increasing of NH_4 concentration. This means that the effect of ammonium on heterocytes formation and their size is variable depending on the strain tested as the effect of nitrate [16, 19]. Larger heterocytes are formed in pulsed regime (NPR) with low number of heterocyte per trichome.

It is easy to differentiate *Cylindrospermopsis* and the other genera of Nostocales by the shape of heterocytes. The shapes of heterocytes are elongated, cylindrical to ellipsoidal, pointed or bluntly pointed at the external end, sometimes curved or little curved, drop-shaped with bluntly or pointed end, mainly in P-starved cultures or under unfavorable conditions. The conical form or drop-shape is the characteristics of unfavourable conditions for growth. The position of heterocytes in trichome is, in most case, terminal at one or both ends of the filament and it is rarely intercalary (Fig. 6). All these forms of heterocytes may be found at the same time in the same culture, i.e. it is not the character that distinguishes different species. What is more *C. africana* and *C. cuspis* have never been recorded in Lake Balaton (the source of the investigated *Cylindrospermopsis*).

In conclusion the morphological features of *C. raciborskii* are highly effected by environmental conditions and nutrient availability. The specifications of *C. africana* and *C. cuspis* overlap with that of *C. raciborskii* accordingly this is no clear character for distinguishing species. A pure culture of a pro- or eukaryote alga growing in continuous cultures is a good method for giving suitable overview on all morphological possibilities of a tested organism [25].

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