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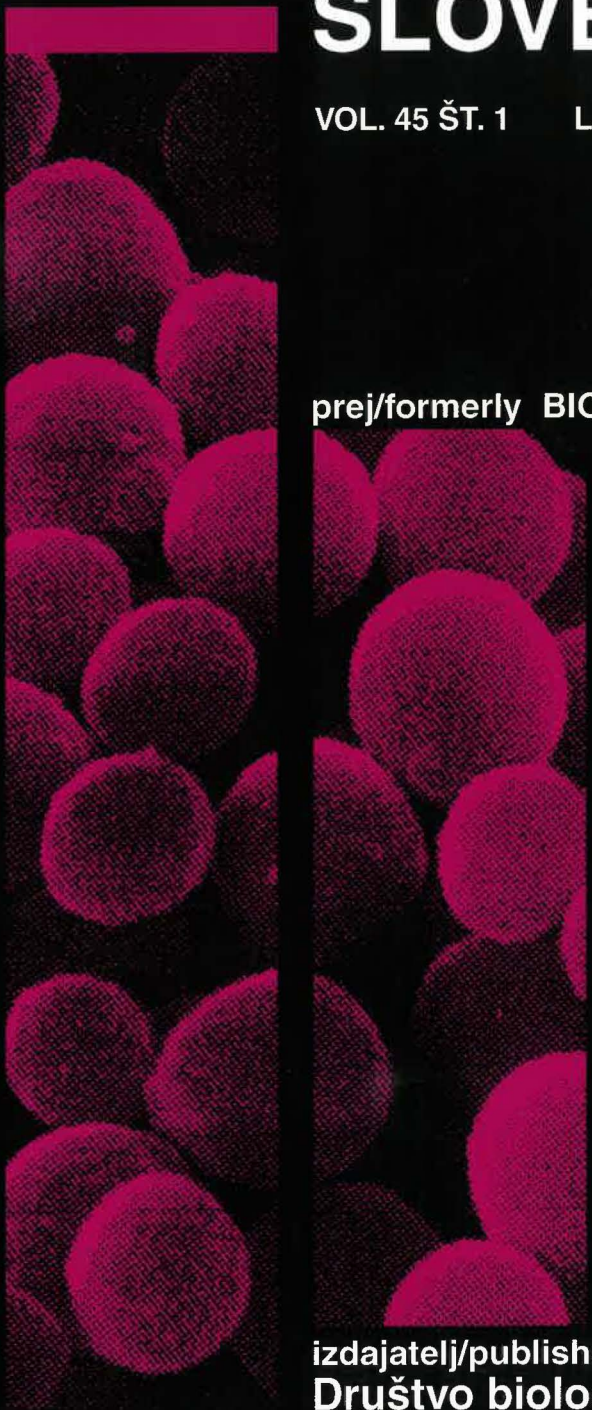

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Identification of physiological races of *Colletotrichum lindemuthianum* occuring in Slovenia

Identifikacija fizioloških ras glive *Colletotrichum lindemuthianum* v Sloveniji

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Abstract. Bean anthracnose, the most important fungal disease of common bean in Slovenia, is caused by a mitosporic fungus *Colletotrichum lindemuthianum* (Sacc. et Magnus) Briosi et Cav. The most appropriate measure to control the disease is growing of resistant cultivars. Breeding for resistance is limited by the existence of several physiological races of the pathogen and continuous development and introduction of new ones. It is necessary to constantly monitor the occurrence and dynamic of physiological races existing in certain area. With this aim, 47 isolates were obtained from several bean accessions in different bean growing areas in Slovenia. The fungus was isolated from infected pods and leaves. A set of 12 internationally accepted differential bean cultivars was inoculated with spore suspension obtained from single spore isolates. Plant reaction was screened following 1 – 10 severity scale. Four physiological races of the pathogen were identified. Using the binary nomenclature system they were assigned to races 23, 55, 103 and 131. The most frequent and widespread were races 55 and 23, named also lambda and delta. The research will continue by screening the autochthonous bean germplasm for susceptibility towards the existing races.

Key words: bean anthracnose, *Colletotrichum lindemuthianum*, common bean, physiological races

Izvleček. Fižolov ožig, ki je pri nas najpomembnejša glivična bolezen fižola, povzroča gliva *Colletotrichum lindemuthianum* (Sacc. et Magnus) Briosi et Cav. Bolezen preprečujemo predvsem z gojenjem odpornih sort fižola. Žlahtnjenje na odpornost proti tej bolezni je težavno zaradi velikega števila fizioloških ras ter nenehnega razvoja in introdukcije novih ras. Zato moramo stalno spremljati pojav in dinamiko fizioloških ras na določenem območju. S tem namenom smo zbrali 47 primerkov glive *C. lindemuthianum* z različnih območij pridelovanja fižola v Sloveniji. Glivo smo izolirali iz okuženih fižolovih strokov in listov. S pridobljenimi izolati smo okužili 12 diferencialnih sort fižola, ki so mednarodno priznane za identifikacijo fizioloških ras te glive. Testne rastline fižola smo okužili s suspenzijo trosov, pridobljeno iz enotrosnih izolatov. Jakost okužbe smo ocenili po desetstopenjski skali. Odkrili smo štiri fiziološke rase glive in jih na podlagi binarnega nomenklaturega sistema identificirali kot rase št. 23, 55, 103 in 131. Najbolj pogosti in razširjeni sta rasi št. 55 in 23, imenovani tudi lambda in delta. V nadaljnjih raziskavah bomo proučili odpornost domačih akcesij fižola na okužbo s slovenskimi rasami glive *C. lindemuthianum*.

Ključne besede: *Colletotrichum lindemuthianum*, fiziološke rase, fižol, fižolov ožig

Introduction

Bean anthracnose is one of the most destructive diseases in bean growing areas world-wide as well as in Slovenia. It is caused by a mitosporic fungus *C. lindemuthianum* (Sacc. et Magnus) Briosi et Cav. The anamorphic state *Glomerella cingulata* (Stonem.) Spauld et v. Schrenk. f. sp. *phaseoli* can be produced in culture in certain pairings of conidial isolates, but has not yet been found in field conditions (BRYSON & al. 1992). *C. lindemuthianum* has a world wide distribution. It has been recorded on various legumes belonging to the genera *Phaseolus, Vicia, Vigna, Glycine, Pisum* and many others (LENNÉ 1992). Among them, *P. vulgaris* is the most important host, followed by *P. acutifolius var. latifolius* and *Vigna unguiculata.* Other legumes are only slightly susceptible (TU 1992).

C. lindemuthianum shows an extensive intraspecific variability and exists in the form of several physiological races. Pathogenic variability among isolates of *C. lindemuthianum* was first observed by BARRUS (1911). He distinguished two races of the fungus (alpha and beta) by testing their pathogenicity towards 139 bean cultivars. This was the first description of physiological races in a plant pathogenic fungus. Later on extensive variation in the fungus has been found and several new races were described in the local populations of *C. lindemuthianum* throughout the world. Race identification was traditionally based on the nomenclature system using letters of the Greek alphabet. Many other nomenclature systems using local codes were also used, thus rendering the possibility of comparing the research results very difficult. Altogether 13 races, named by letters of Greek alphabet, were described till 1994 and many more were classified using local codes (MELOTTO & al. 2000).

Bean anthracnose affects all the above ground parts of beans causing dark brown depressed lesions. Affected leaves and stems bear brown lesions that can rapidly spread and girdle the plant. Symptoms are the most conspicuous on pods: rusty brown spots occur first, then sunken dark brown, eye-shaped lesions with brown to reddish edges develop. Black specks (acervuli) occur on the lesions and pink spore masses ooze from them in humid conditions. The disease is transmitted and spread mostly by infected seed. Seed treatment and production of clean seed, crop rotation and sanitation can significantly reduce the extent of the disease. Nevertheless, outbreaks of anthracnose still occur, especially in areas with an extensive use of susceptible local bean accessions. The intensity of the disease depends on weather conditions and can reach the extent of an epidemic in the years with high relative humidity, frequent precipitation and moderate temperature.

The most appropriate measure to control the disease is growing of resistant cultivars. Breeding for resistance against *C. lindemuthianum* has been practised since the beginning of the last century, when disease resistance was demonstrated in certain bean cultivars (BARRUS 1911, 1918). Eight independent dominant resistance genes (*Co*-1 to *Co*-8) were described in common bean (ALZATE-MARIN & al. 1997, YOUNG & al. 1998, GEFFROY & al. 2000). The use of specific resistance genes has not always provided a durable resistance due to the continuous development of new physiological races which are capable of overcoming the resistant germplasm. When selecting for resistance in a particular region, the breeder should carefully choose a gene pair that would confer resistance to all races known in that region (KELLY & MIKLAS 1999).

The aim of our study was to investigate the pathogenic variability of the pathogen and identify the physiological races existing in the bean growing areas in Slovenia.

Materials and methods

Fungal isolates

47 samples were collected from several bean accessions in different bean growing areas in Slovenia. The fungus was isolated from infected pods and leaves on potato dextrose agar (PDA). Sporulation was induced by growing isolates on sterilised bean pods. Single spore isolates were obtained by

spreading spore suspension on PDA plates and isolating individual germinating spores. To prevent a degeneration of single spore isolates during storage, spore suspension of single spore isolates was fixed on sterile filter papers, desiccated on sterilised silica-gel and stored at - 20 °C (FERREIRA & FUEYO 2001).

Differential bean cultivars

Physiological races of the fungus were identified by screening the disease severity (resistant or susceptible reaction) on a selected group of bean varieties - differentials. The reaction of differential cultivars strongly depends on infection techniques, experimental conditions, on host genotype and pathogenicity of the fungal isolate. Different bean cultivars, mostly local ones, were used as differentials and different reaction schemes were applied for assessing the disease severity. Several attempts have been made to select an international set of differential cultivars and to uniform the classification of races. Differential cultivars should be genetically uniform with clear resistant or susceptible reaction to all isolates of the fungus (CHARRIER & BANNEROT 1970, DRIJFHOUT & DAVIS 1989). In 1991, a set of 12 differential bean cultivars was internationally accepted and a binary nomenclature system for identification of *C. lindemuthianum* races was proposed (PASTOR - CORRALES 1991). Using this system, each race is identified by a number, obtained by summing the binary value of all differential cultivars showing a susceptible reaction. Among differential cultivars, four represent the Andean germ plasm (Perry Marrow, Michigan Dark Red Kidney, Kaboon and Widusa) and the others - the Middle American germ plasm (Michelite, Cornell 49242, Mexico 222, PI 207262, To, Tu, Ab136 and G2333).

The seeds of differential bean cultivars used in our study were obtained from CIAT, Columbia. The seeds were surface sterilised with 1 % sodium hypochlorite, left to germinate on moist filter paper and planted in sterilised sand (four seeds per pot). They were kept in greenhouse for two weeks until the development of primary leaves.

Infection trials

A single spore colony of each isolate was grown on PDA for 14 days prior to inoculation. Spores were then scrapped from the plates and used for the preparation of spore suspension. Spore concentration was adjusted to 10^6 - 10^7 per ml prior to inoculation of test plants. Plants with fully expanded primary leaves were infected. Both sides of leaves and stems were inoculated by brushing following the method of TU & AYLESWORTH (1980). Four plants of each cultivar were infected with each isolate and the whole experiment was repeated two to three times. Altogether 1690 inoculations were made. The plants were kept in a growth chamber at the temperature 20 °C and 14 h of light. During the first two days of incubation they were covered with transparent plastic bags to maintain a 100 % relative humidity. The disease expression was scored 8 days after the inoculation. The plant reaction was evaluated qualitatively (resistant or not) and quantitatively using ten- point severity scale (0 - 9). Plants showing no infection or small necrotic lesions originating from hypersensitive reaction, were assigned resistant (score 0 –3), the rest were considered susceptible (score 4 - 9).

Results and discussion

Isolates of *C. lindemuthianum* from *Phaseolus vulgaris* grown in Slovenia were assigned to four physiological races based on their pathogenicity towards 12 differential bean cultivars (Table 1). Most of the isolates were pathogenic to differential cultivars originating from Andean gene pool. Among them, the cultivar Michigan Dark Red Kidney was the most liable to infection and showed a highly susceptible reaction to all isolates. By contrast differential cultivars originating from Middle American gene pool were relatively resistant with the exception of the cultivar Michelite, which showed a moderately susceptible reaction to most of the isolates.

Using the binary nomenclature system the races of *C. lindemuthianum* from Slovenia can be identified as races 23, 55, 103 and 131. The majority of isolates (25) represented race 55, 16 isolates represented race 23, and only 6 isolates represented races 103 and 131.

Table 1: Reaction of differential bean cultivars to races of *C. lindemuthianum* from Slovenia
Tabela 1: Reakcija diferencialnih kultivarjev fižola pri okužbi s slovenskimi rasami glive *C. lindemuthianum*

Race	Differential bean cultivars											
	1	2	4	8	16	32	64	128	256	512	1024	2048
131	S_{5-7}	S_9	R_0	R_0	R_3	R_3	R_2	S_{4-5}	R_3	R_0	R_2	R_0
103	R/S_4	S_9	S_9	R_0	R_3	S_8	R/S_4	R_2	R_3	R_0	R_2	R_0
23	S_7	S_9	S_9	R_0	S_8	R_{2-3}	R_2	R_1	R_3	R_0	R_2	R_0
55	S_7	S_9	S_9	R_0	S_8	S_{5-7}	R_2	R_2	R_2	R_0	R_2	R_0

Legend: Differential bean cultivars: 1- Michelite, 2 - Michigan Dark Red Kidney, 4 - Perry Marrow, 8 - Cornell 49242, 16 - Widusa, 32 - Kaboon, 64 - Mexico 222, 128 - PI 207262, 256 - TO, 512 - TU, 1024 - AB 136, 2048 - G 2333; R - resistant reaction, S - susceptible reaction, numbers indicate severity of symptoms (ten- point severity scale).
Legenda: Diferencialni kultivarji fižola: 1- Michelite, 2 - Michigan Dark Red Kidney, 4 - Perry Marrow, 8 - Cornell 49242, 16 - Widusa, 32 - Kaboon, 64 - Mexico 222, 128 - PI 207262, 256 - TO, 512 - TU, 1024 - AB 136, 2048 - G 2333; R - odporen, S - občutljiv, številke kažejo jakost okužbe po desetstopenjski skali

As has already been mentioned, many different systems of race classification are used in the studies of pathogenic variability in *C. lindemuthianum*. The results are difficult to compare since different bean cultivars are used as differentials. Nevertheless, comparing our results with other studies of *C. lindemuthianum* race composition in Europe in the past decades (CHARRIER & BANNEROT 1970, DRIJFHOUT & DAVIS 1989, GOTH & ZAUMEYER 1965, KRUGER & al. 1977, YERKES & ORTIZ 1956) we can conclude that races 55 and 23 fully correspond with the races lambda and delta. The race lambda has been first reported in Europe in 1974 from the Netherlands and the race delta in 1953 from Germany (HUBBELING 1974, FRANDSEN 1953). Both races are very virulent, showing high level of pathogenicity towards many bean cultivars. The minority of isolates tested in our study belonged to races 103 and 131. They partly correspond with the races gamma and beta. Their reaction to differential cultivars, especially to the cultivar Michelite, slightly differs from the reaction described in other studies; Michelite was moderate to susceptible in our study, while it was resistant in most other studies (ALAM & RUDOLPH 1993, FERNÁNDEZ & AL. 2000, GOTH & ZAUMEYER 1965, YERKES & ORTIZ 1956).

The existence of a large number of *C. lindemuthianum* races and a continuous emergence of the new ones are very important aspects of the control of bean anthracnose. It is necessary to constantly monitor the race composition in a certain area and screen the local bean germplasm for susceptibility towards the existing races. With this aim we intend to infect 27 local bean accessions with isolates representing all the four races of the pathogen occurring in Slovenia. Preliminary results of infection with three isolates of the delta race revealed that only 4 local accessions were resistant to the anthracnose pathogen, the rest were susceptible with a level of infection ranging from 5 to 8.

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tra region of the natural conjugative *Escherichia coli* plasmid pRK100 is F-like

Regija *tra* naravnega konjugativnega plazmida pRK100 bakterije *Escherichia coli* je podobna plazmidu F

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Abstract. The aim of the presented study was to identify the similarity of the pRK100 *tra* region with *tra* regions of other conjugative plasmids of *Enterobacteriaceae*. For this purpose several *tra* genes were amplified with PCR and the nucleotide sequences of the obtained PCR products were determined. The pRK100's nucleotide sequences were compared to the nucleotide sequences deposited in GenBank and the nucleotide divergence between them was calculated. The obtained results clearly demonstrated, that the *tra* region of pRK100 is the most similar to the *tra* region of plasmid F.
Key words: conjugative plasmid, *tra* region, *Enterobacteriaceae*.

Izvleček. Cilj raziskave je bil ugotoviti podobnost regije *tra* plazmida pRK100 z regijami *tra* drugih konjugativnih plazmidov enterobakterij. V ta namen smo več genov regije *tra* plazmida pRK100 pomnožili v PCR in določili nukleotidno zaporedje dobljenih produktov PCR. Nukleotidna zaporedja plazmida pRK100 smo primerjali z drugimi, deponiranimi, nukleotidnimi zaporedji in izračunali nukleotidno divergenco. Dobljeni rezultati so jasno pokazali, da je regija *tra* plazmida pRK100 najbolj podobna regiji *tra* plazmida F.
Ključne besede: konjugativen plazmid, regija *tra*, *Enterobacteriaceae*.

Introduction

Conjugative plasmids are extrachromosomal elements, that can promote their own DNA transfer, as well as of co-resident plasmids or even chromosomal DNA from a donor to a recipient cell in a process named conjugation (Firth & al. 1996). All information needed for this process is encoded in a large, approximately 30 kb long, plasmid region, often denoted as the *tra* region. Conjugative plasmids facilitate the exchange and spread of resistances to antibiotics, chemicals, virulence factors and metabolic properties.

One of the best studied *tra* regions is that of the *Escherichia* coli plasmid F (Fig. 1) The *tra* region of plasmid F contains approximately 40 genes that are organised into three operons with a complex network of regulation of gene expression (Frost & al. 1994). The genes of the F-plasmid *tra* region can be divided, according to function, into 5 groups: i) regulatory genes (*finP, traJ, traY,* and *finO*); ii) genes for pilus biogenesis (*traA, traL, traE, traK, traB, traV, traC, trbI, traW, traU, trbC, traF, traQ,*

traG, traH, traX); iii) genes for DNA metabolism (*traM, traY, traD, traI*); iv) genes for aggregate stabilisation (*traN, traG*); and v) genes for surface exclusion (*traS, traT*) (Firth & al. 1996).

Several conjugative plasmids of *Enterobacteriaceae* have been found to harbour F-like *tra* regions: pColV-K30, P307, R100, R1, pSLT. Even though these plasmids have many similarities at the level of nucleotide sequences, differences in regulation of conjugation are observed. For example: plasmid R1 has two promoters upstream of the *traJ* gene, while plasmid F has only one promoter (Dempsey 1994). Therefore, characterisation of different conjugative plasmids and their *tra* regions is of interest.

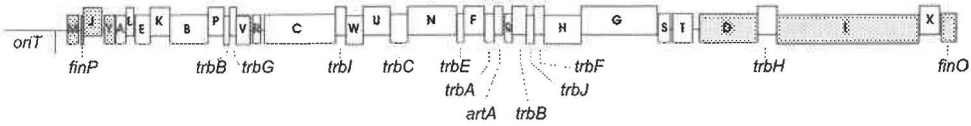

Figure 1: Physical and genetic map of the F-plasmid *tra* region. Genes of the F *tra* region are depicted. To clearly show the region of a gene, some boxes representing genes are offline. Capital letters are shortened abbreviations for the genes named *tra*. The boxes designating the pRK100 genes analysed in this study are filled. The figure is based on Frost & al. 1994.
Slika 1: Razpored genov *tra* plazmida F. Prikazani so geni *tra* plazmida F. Kvadrati, ki označujejo gene, katerih nukleotidna zaporedja smo analizirali, so zapolnjeni. Slika je pripravljena po Frost & al. 1994.

In the presented study the *tra* region of the natural conjugative plasmid pRK100 was characterised with regard to similarity with *tra* regions of related plasmids. pRK100 is a ~145-kb plasmid isolated from a uropathogenic *Escherichia coli* strain and it has been partially characterised (Ambrožič & al. 1998). It is a member of the IncF incompatibility group and encodes two antibiotic resistances, ampicillin and tetracycline, two colicins, ColV and ColIa, and the aerobactin iron uptake system (Žgur-Bertok & al. 1990).

For the purpose of defining the similarity of pRK100's *tra* region, PCR products of several *tra* genes were amplified and their nucleotide sequences were determined. The obtained (partial) nucleotide sequences of genes *traM, finP, traJ, traY, traD, traI,* and *finO* were compared with the nucleotide sequences of the same genes of plasmids F, pColV-K30, P307, R100, R1 and pSLT and the nucleotide divergence between pRK100 and the other plasmids was determined. From the obtained results it can be concluded that the *tra* region of pRK100 is most similar to the *tra* region of plasmid F.

Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are presented in Tab. 1. Bacteria were grown in Luria-Bertani (LB) medium with aeration at 37°C. Ampicillin (Ap, 100 µg/ml) and tetracycline (Tc, 10 µg/ml) were added to the growth media, when appropriate.

Table 1: Bacterial strains and plasmids
Tabela 1: Bakterijski sevi in plazmidi

Strain or DNA	Relevant features	Reference or source*
Strains		
HB101	*hsdR hsdM recA13 supE44 leuB6 lacZ proA2*	D. Ehrlich[1]
CL225	HB101 harbouring pRK100	Ambrožič & al. 1998
DH5α	Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	BRL Life Technologies
Plasmids		
pGEM-T Easy	T-vector for cloning of PCR products; Ap^r	Promega
pRK100	natural plasmid; Ap^r, Tc^r	Ambrožič & al. 1998

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General DNA manipulation techniques

Plasmid DNA isolation, ligation and transformation experiments were performed using standard methods (Sambrook & al. 1989). Restriction endonuclease digestions were carried out as specified by the manufacturer (Promega, Boehringer). DNA fragments were isolated from agarose gels using the QIAquick Gel Extraction kit (Qiagen). DNA sequencing was performed using a dye rhodamine terminator cycling reaction and an ABI PRISM™ 310 Genetic Analyzer automated sequencer and ABI PRISM™ software.

Polymerase chain reaction

The primers used for the various PCR reactions are presented in Tab. 2. The polymerase chain reactions (PCR) were performed in a 50 µl PCR reaction mixture with 20 pmol of the two primers, 5 µl ligation mixture, 0.2 mM of dNTP mixture (Pharmacia), 0.625 U Taq DNA polymerase (Promega) and 1× PCR buffer (Promega). The PCR programs used in this study are listed in Tab. 2. Each PCR amplification program started with a prolonged denaturation (94°C – 4 min) step before 1st cycle, and ended with a prolonged extension step (72°C – 10 min) after the last cycle. The pGEM-T Easy system (Promega) was used for cloning of PCR products.

Table 2: Oligonucleotide primers and PCR programs
Tabela 2: Oligonukleotidni začetniki in programi verižne reakcije s polimerazo (PCR)

Primer	Nucleotide sequence	PCR program
Oligonucleotide primers for *traM*, *finP*, *traJ* PCR:		
FinP-1	5'-TATTGAGAAGCGTCGACAGG-3'	(94°C-1:00, 55°C-1:00,
FinP-2	5'-TGACGAACATGAGCAGCATC-3'	72°C-1:00) 30×
for *traY* PCR:		
TraY-f[1]	5'-GGAATTCAAGATTTGGTACACGTTCTGC-3'	(94°C-1:00, 63°C-
TraY-r[1]	5'-GGAATTCCTTCCTCTTTATCTGCCTCCC-3'	1:00, 72°C-2:00) 30×
for *traD* PCR:		
TraD-f[1]	5'-GGAATTCCAGATTGCGTCCATGCGTATCC-3'	(94°C-1:00, 63°C-1:00,
TraD-r[1]	5'-GGAATTCATCACCACACATATCACCGCGC-3'	72°C-1:00) 30×
for *traI* PCR:		
TraI-1	5'-ACAGCGAATATACGTGACGG-3'	(94°C-0:30, 57°C-0:30,
FinO-4	5'-CGTGGTGACATTGATGATGG-3'	72°C-3:00) 30×
for *finO* PCR:		
FinO-f[1]	5'-GGAATTCGAAGCGACCGGTACTGACACTG-3'	(94°C-1:00, 63°C-1:00,
FinO-r[1]	5'-GGAATTCGCCTGAAGTTCTGCCTTTATCCG-3'	72°C-2:00) 30×

[1] Nucleotide sequence is based on Boyd & al. 1996
[1] Nukleotidno zaporedje z ozirom na Boyd & al. 1996

Sequence analysis

DNA sequences were compiled and analysed using the CLUSTAL W (Thompson & al. 1994) program for sequence alignment and the program DNADIST in the PHYLIP package (Felsenstein 1993, Felsenstein 1989) for calculating distance matrixes.

Results

The compared nucleotide sequence of pRK100 *traM* is most similar to *traM* of plasmids F and pColV-K30

The *traM* gene of F-like plasmids is approximately 380 bp long. Our obtained nucleotide sequence of the pRK100 *traM* gene is partial, covering 308 nucleotides (GenBank accession number AF237698). Subsequently, in nucleotide sequence analysis the obtained 308 nucleotides were compared with

corresponding sequences of related plasmids. The phylogenetic distance between the obtained *traM* nucleotide sequence and *traM* of pColV-K30 and plasmid F is the smallest, namely 0.0098 (Fig. 2A). On the basis of the calculated nucleotide divergence, we can conclude, that the *traM* nucleotide sequence of pRK100 is most similar to *traM* of F and pColV-K30.

The compared nucleotide sequence of pRK100 *finP* is identical to *finP* of plasmid F

The *finP* gene of the F-like plasmids is approximately 80 bp long and the full length pRK100 *finP* gene sequence (GenBank accession number AF237698) was compared with corresponding sequences of related plasmids. Since there is no nucleotide divergence between pRK100 *finP* and F *finP* (Fig. 2B), the *finP* of pRK100 is completely the same as the *finP* of plasmid F.

The compared nucleotide sequence of pRK100 *traJ* is most similar to *traJ* of plasmid F

The *traJ* gene of F-like plasmids is 600-700 bp long. Our obtained nucleotide sequence of pRK100 *traJ* gene is partial, covering approximately one half of the gene (363 bp) (GenBank accession number AF237698). Therefore, in nucleotide sequence analysis the obtained 363 bp were compared with corresponding sequences of related plasmids. The phylogenetic distance between the obtained *traJ* nucleotide sequence and *traJ* of plasmid F is the smallest, only 0.0028. The distance to *traJ* of plasmid pColV-K30 is also very small, only 0.0055 (Fig. 2C). We can conclude that, the *traJ* nucleotide sequence of pRK100 is most similar to *traJ* of plasmid F.

The compared nucleotide sequence of pRK100 *traY* is most similar to *traY* of plasmids F and pColV-K30

The *traY* genes of F-like plasmids can be one of two lengths, approximately 230 bp, and probably due to duplication (Maneewannakul & al. 1996), approximately 400 bp. Our obtained nucleotide sequence of the pRK100 *traY* gene is partial, covering 326 nucleotides (GenBank accession number AF237695). Due to the larger size, we can conclude that, the pRK100 *traY* is of the duplicated type. In our sequence analysis the obtained 326 bp were compared with corresponding sequences of related plasmids. The compared nucleotide sequences of pRK100, pColV-K30 and plasmid F were completely identical with no nucleotide divergence among them (Fig. 2D).

The compared nucleotide sequence of pRK100 *traD* is most similar to *traD* of plasmid F

The *traD* gene of F-like plasmids is approximately 2200 bp long. Our obtained nucleotide sequence of pRK100 *traD* gene is partial, covering 574 bp (GenBank accession number AF237693). Therefore, in nucleotide sequence analysis the obtained 574 nucleotides were compared with corresponding sequences of related plasmids. The phylogenetic distance between the obtained *traD* nucleotide sequence and *traD* of plasmid F is the smallest, only 0.0359 (Fig. 2E). We can conclude, that the *traD* nucleotide sequence of pRK100 is most similar to *traD* of plasmid F.

The compared nucleotide sequence of pRK100 *traI* is most similar to *traI* of plasmid R100

The *traI* gene of F-like plasmids is approximately 5200 bp long. Our obtained nucleotide sequence of pRK100 *traI* gene is partial, covering only 258 bp (GenBank accession number AY230887). In the nucleotide sequence analysis the obtained 258 bp were compared with corresponding sequences of related plasmids. The phylogenetic distance between the obtained *traI* nucleotide sequence and *traI* of plasmid R100 is the smallest, the nucleotide divergence is 0.0237 (Fig. 2F). The *traI* nucleotide sequence of pRK100 is therefore, most similar to *traI* of plasmid R100.

The compared nucleotide sequence of pRK100 *finO* is most similar to *finO* of plasmid F

The *finO* gene of the F-like plasmids is approximately 560 bp long. The obtained pRK100 *finO* gene sequence is partial, encompassing 486 nucleotides (GenBank accession number AF237696) and these were compared with corresponding sequences of related plasmids. The smallest nucleotide divergence, 0.0316, was found to be between pRK100 *finO* and F *finO* (Fig. 2G). Therefore, we can conclude that *finO* of pRK100 is most similar to *finO* of plasmid F.

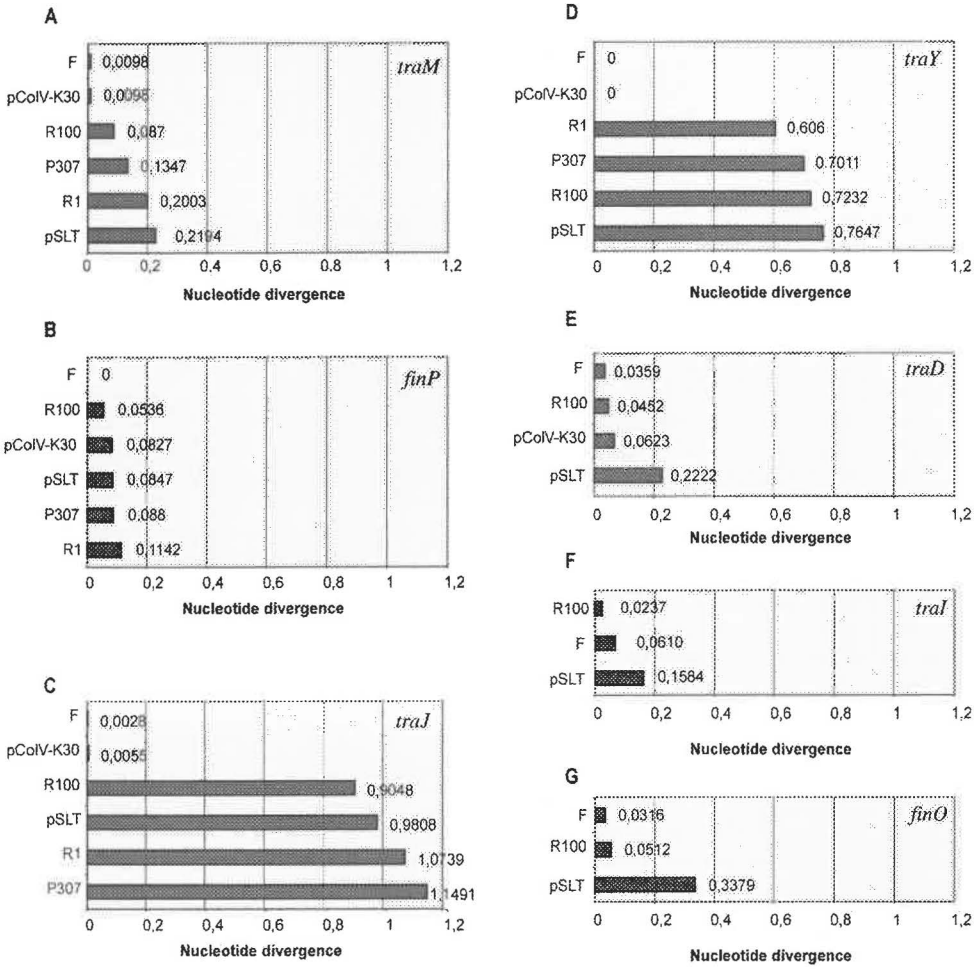

Figure 2: Nucleotide divergence of the obtained pRK100 *tra* sequences and related plasmid sequences. The nucleotide sequences of pRK100 related plasmids used in the sequence analysis of *tra* pRK100 nucleotide sequences are deposited in GenBank under the following accession numbers: F-plasmid - U01159; pColV-K30 - AF237697 (*traM, finP, traJ*), AF237694 (*traY*), AF237692 (*traD*); P307 – M62986; R100 – AP000342; R1 – M19710 and pSLT – AE006471.
Nucleotide divergence, as the measurement of phylogenetic nucleotide distance, was calculated according to the Kimura-2 parameter. Nucleotide divergences between pRK100 sequences and sequences of other related plasmids are plotted: Panel A – nucleotide divergence of *traM*, panel B – nucleotide divergence of *finP*, panel C – nucleotide divergence of *traJ*, panel D – nucleotide divergence of *traY*, panel E – nucleotide divergence of *traD*, panel F – nucleotide divergence of *traI* and panel G – nucleotide divergence of *finO*.
Slika 2: Divergenca nukleotidnih zaporedij genov *tra* plazmida pRK100 in nukleotidnih zaporedij sorodnih plazmidov. Nukleotidna zaporedja plazmidov sorodnih pRK100, katera smo uporabili v analizi zaporedij *tra* genov pRK100, so shranjena v GenBank in označena s sledečimi številkami: Plazmid F - U01159; pColV-K30 - AF237697 (*traM, finP, traJ*), AF237694 (*traY*), AF237692 (*traD*); P307 – M62986; R100 – AP000342; R1 – M19710 in pSLT – AE006471.

Nukleotidna divergenca kot izračun filogenetske drugačnosti nukleotidov je bila preračunana z uporabo parametra Kimura-2. Divergence nukleotidnih zaporedij plazmida pRK100 in nukleotidnih zaporedij sorodnih plazmidov so prikazane: panel A – nukleotidna divergenca gena *traM*, panel B – nukleotidna divergenca gena *finP*, panel C – nukleotidna divergenca gena *traJ*, panel D – nukleotidna divergenca gena *traY*, panel E – nukleotidna divergence gena *traD*, panel F – nukleotidna divergenca gena *traI* in panel G – nukleotidna divergence gena *finO*.

Discussion

In order to characterise the *tra* region of pRK100, nucleotide sequences of seven different *tra* region genes, amplified by PCR, were determined and compared with nucleotide sequences of related plasmids. The chosen *tra* region genes were: *traM, finP, traJ, traY* from one end the and *traD, traI, finO* from the other end of the pRK100 *tra* region. The obtained pRK100 nucleotide sequences were compared with nucleotide sequences of the following F-like plasmids: plasmid F, pColV-K30, P307 and R1 from *Escherichia coli*, R100 from *Shigella flexneri* and pSLT from *Salmonella typhimurium*.

The choice of related plasmids was based on the fact, that pRK100 seems to be an F-like plasmid, which evolved from at least two F-like plasmids. In our previous work it was discovered, that pRK100 is an IncFI plasmid harbouring a RepFIB (Ambrožič & al. 1998) and RepFIIA replication region. The RepFIB replication region is similar to the RepFIB replication region of plasmid F, pColV-K30, P307, and the RepFIIA replication region is similar to R100 (our unpublished data). pRK100 also encodes colicin V and the aerobactin uptake system as does plasmid pColV-K30 (Ambrožič & al. 1998). Further, it also carries IS*1*, which is also present on pColV-K30 and on plasmid R100. Apart from IS*1*, pRK100 also encodes IS2 and IS*3* insertion sequences (our unpublished data), which are also present on plasmid F, but not on R100.

A similar melange also emerges from results presented in this paper. The *traM* gene of pRK100 has the same nucleotide divergence with *traM* of F and *traM* of pColV-K30; the *finP* gene of pRK100 is exactly the same as *finP* of F; *traY* of pRK100 has the same nucleotide sequence as *traY* of F but also the same sequence as *traY* of pColV-K30; pRK100 *traJ, traD* and *finO* are most similar to *traJ, traD* and *finO* of plasmid F; but *traI* of pRK100 is most similar to *traI* from R100. On the basis of our results we can therefore conclude that, the *tra* region of pRK100 is F-like and it is most similar to the *tra* region of plasmid F.

Even though all the plasmids, incorporated into this study, are known to be F-like plasmids, nucleotide sequence divergence among the different genes is not the same, some genes are more conserved than others. For example, the regulatory genes *traJ* and *traY* exhibit greater differences, than for example the *traD* gene, whose product is involved in transmembrane conveyance of nucleic acids (Firth & al. 1996). Plasmid genes are mosaic in structure due to multiple recombination events between diverse ancestral genes (Boyd & al. 1996). Conjugation by introducing horizontally transferred DNA into cells increases the opportunity of different plasmids to meet and exchange genetic information. Knowledge of plasmid structure and transfer genes is necessary to develop efficient means to reduce plasmid transfer and dissemination of antibiotic resistances as well as bacterial virulence factors.

Conclusions

To summarise and conclude:

1. different *tra* genes of pRK100 show different levels of nucleotide divergence with different related plasmids;
2. the *tra* region of pRK100 is F-like;
3. the pRK100 *tra* region is pronouncedly most similar to the *tra* region of plasmid F
4. the pRK100 *tra* region is mosaic.

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Harmful cyanobacterial blooms in Slovenia – Bloom types and microcystin producers

Škodljiva cianobakterijska cvetenja v Sloveniji – Tipi cvetenj in proizvajalci mikrocistinov

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Abstract. Up to now, research on cyanobacteria and their biologically active substances has been directed principally towards their harmful effects on humans, and little has been done to elucidate their ecological role. In order to understand better the biological success of cyanobacterial blooms, and in order to be able to compare the results of different scientific investigations, we must find and agree on a definition of the phenomenon. We propose a definition of harmful cyanobacterial blooms based on the OECD boundary system of eutrophication with the addition of phycocyanin values. We have found a direct linkage between the trophic conditions in the water-bodies and the frequency of formation of cyanobacterial blooms.

Specific toxic species and their strains have been studied intensively. However, in order to elucidate the mechanisms that enable cyanobacteria to overtake eutrophic water bodies we must change our approach. Cyanobacterial blooms should not be treated merely as different species or strains but as superorganisms. It is their intraspecific diversity that permits cyanobacteria to be successful in a variable water environment. We here focus attention on microcystin producers and microcystins as an adaptation to the limited light conditions, which arise in cyanobacterial blooms. The conclusions are illustrated with some data from surface water-bodies in Slovenia.

Key words: cyanobacteria, blue-green algae, harmful bloom, microcystins, eutrophication.

Izvleček. Raziskave cianobakterij in biološko aktivnih snovi, ki jih proizvajajo so bile do sedaj usmerjene predvsem na proučevanje škodljivih učinkov na človeka. Zelo malo je bilo storjenega v smeri preverjanja njihove ekološke vloge. Za boljše razumevanje razvojnega uspeha cianobakterijskih cvetenj in da bi bili sploh sposobni primerjati rezultate različnih znanstvenih raziskav moramo najti najprej definicijo tega pojava. Predlagamo opredelitev škodljivih cianobakterijskih cvetov, ki temelji na OECD razmejitvenem sistemu evtrofikacije z dodanimi vrednostmi za fikocianine. Ugotovili smo neposredno zvezo med trofičnim stanjem vodnih teles in pogostostjo pojavljanja cianobakterijskih cvetov.

Številne toksične cianobakterijske vrste in soje so že podrobno proučevali. Vendar za boljše razumevanje mehanizmov, ki jim omogočajo prevlado v evtrofnih vodnih

telesih, moramo spremeniti naš pristop. Cianobakterijske cvetove ne moremo obravnavati le kot zmesi različnih vrst in sojev, temveč kot superorganizme. Prav njihova intraspecifična raznovrstnost jim omogoča uspeh v spremenljivem vodnem okolju. Našo pozornost bomo osredotočili na tiste cianobakterije, ki so sposobne proizvajati mikrocistine in na mikrocistine kot možno prilagoditev na omejene svetlobne razmere, kakršne nastopajo ob cvetenjih. Zaključki smo podkreplili s podatki o vodnih telesih v Sloveniji.

Ključne besede: cianonbakterije, modrozelene-alge, škodljivo cvetenje, mikrocistini, evtrofikacija.

Introduction

The deterioration of surface water quality is becoming one of the main problems facing humanity in the near future. Some of the most evident consequences are the dense cyanobacterial blooms that further contribute to poor quality and the need for expensive treatment of affected waters (*e.g.* FERGUSSON et al. 1996). Cyanobacteria are also able to produce a wide range of toxic metabolites – microcystins (MC) being the most abundant – that additionally reduce water utilization (CARMICHAEL 1992).

Contemporary scientific concern is based primarily on an anthropocentric point of view, and this is the main reason why cyanobacteria and microcystins have been given serious attention. They are frequently the cause of health problems in humans and livestock (CARMICHAEL 1992, LAHTI 1997). As a consequence, microcystins have been treated only as toxins, and little attention has been paid to their ecological role.

The universal view of microcystins as hepatotoxins does not take into account the fact that cyanobacteria were present more than two billion years ago, long before the appearance of higher organisms (SCHOPF 1993, SCHOPF 2000). The assumption that cyanobacteria in their early stage of evolution already produced microcystins is, of course, speculative but on the other hand it is unlikely that they developed these complex substances just as a defence mechanism against potential predators. The overall logic of evolution is based principally on egoism rather than aggressiveness, and we can therefore expect organisms to be stimulated to produce substances that favour better adaptation, rather than cause harm to rivals. The implication of this statement will be explained later, once we have defined the harmful cyanobacterial bloom where the mass production of microcystins frequently takes place.

Material and methods

Field sampling

Eighty-four surface water bodies were regularly inspected for cyanobacterial blooms over an eight years period (1994-2001). Three different samples were taken at each location where the blooms occurred.

1) Water samples. Samples for chlorophyll *a* determination were taken beneath the water surface in order to avoid the surface bloom and from the bloom itself. The extraction was performed with hot methanol according to VOLLENWEIDER (1974).
2) Net samples. Qualitative 25 mesh net samples were taken as a vertical profile, preserved in 5% formaldehyde and analysed for phytoplankton species composition and their abundance rated using three categories: present, subdominant and dominant based on their relative biomass (ORLIK 1981)

3) Bloom samples. Cyanobacterial bloom samples were collected by skimming across water surface with a 25 µm plankton net for toxin analysis. We separated larger particles and zooplankton by using different sieves. The samples were then concentrated by placing the material in glass cylinders under natural light. In this way, cell buoyancy was increased and the cyanobacteria floated to the surface.

Cyanobacterial species analysis

The species were identified with the use of an inverted microscope according to Komarek (1958, 1991), Starmach (1966) and Hindak (1978).

Microcystin analysis

The lyophilised bloom material processed according to HARADA et al. (1988), as described elsewhere (SEDMAK & KOSI 1997a). The toxic fractions separated using HPLC were estimated by comparison of the retention times, spectra and peak areas of the standard microcystins.

Results and discussion

Bloom definitions

A classic bloom definition is that proposed by LINDHOLM (1994), who describes blooms as »remarkable phytoplankton maxima, in which organisms are highly concentrated and often almost monospecific«.

The expression "bloom" is still poorly defined. Usually it describes a phytoplankton biomass that is significantly higher than the average found in a water body. As the blooms are usually composed of one or two plankton species they are accordingly named after the dominant phytoplankton species. Additionally there is another problem derived from the varying cell volume of the phytoplanktonts. Which organism is actually blooming, the minute but very frequent one, or the bigger although seldom appearing? The representation only by cell concentration cannot reflect the real situation, since cells of voluminous species never occur as frequently as the minute ones even if they constitute the bulk of biomass. In freshwaters with high productivity we are confronted with different blooms such as green algal blooms, diatom blooms, cyanobacterial blooms, etc.

The fact that bloom forming cyanobacteria are able to produce a broad range of biologically active substances (CARMICHAEL 1994) that are harmful to humans as well as to other organisms, has created the need for a better definition of this phenomenon.

Waters rich in nutrients - eutrophic waters - are favourable to the mass development of phytoplankton. There is an indisputable linkage between water eutrophication and cyanobacterial bloom formation. Since in oligotrophic waters the cyanobacterial bloom forming species are extremely rare or even absent, we have based our definition of harmful cyanobacterial blooms on the OECD boundary system of eutrophication (ANON. 1982).

Table 1: OECD boundary values for trophic categories supplemented with proposed phycocyanin values.
Tabela 1: Mejne OECD vrednosti za trofične kategorije voda z dodanimi predlaganimi vrednostmi za fikocijanine.

Trophic category	**P** (mg/m^3)	***Chl.*** (mg/m^3)	Max. ***chl.*** (mg/m^3)	***Phycocy.*** (mg/m^3)	*Max.* ***Phycocy.*** (mg/m^3)	***Secchi*** (m)	Min. ***Secchi*** (m)
Ultra-oligotrophic	≤ 4	≤ 1	≤ 2.5	≤ 1	≤ 2.5	≥ 12	≥ 6
Oligotrophic	≤ 10	≤ 2.5	≤ 8	≤ 2.5	≤ 8	≥ 6	≤ 3
Mesotrophic	10 – 30	2.5 - 8	8 - 25	2.5 - 8	8 - 25	6 - 3	3 – 1.5
Eutrophic	35 - 100	8 - 25	25 - 75	8 - 25	25 - 75	3 – 1.5	1.5 – 0.7
Hypertrophic	≥ 100	≥ 25	≥ 75	≥ 25	≥ 75	≤ 1.5	≤ 0.7

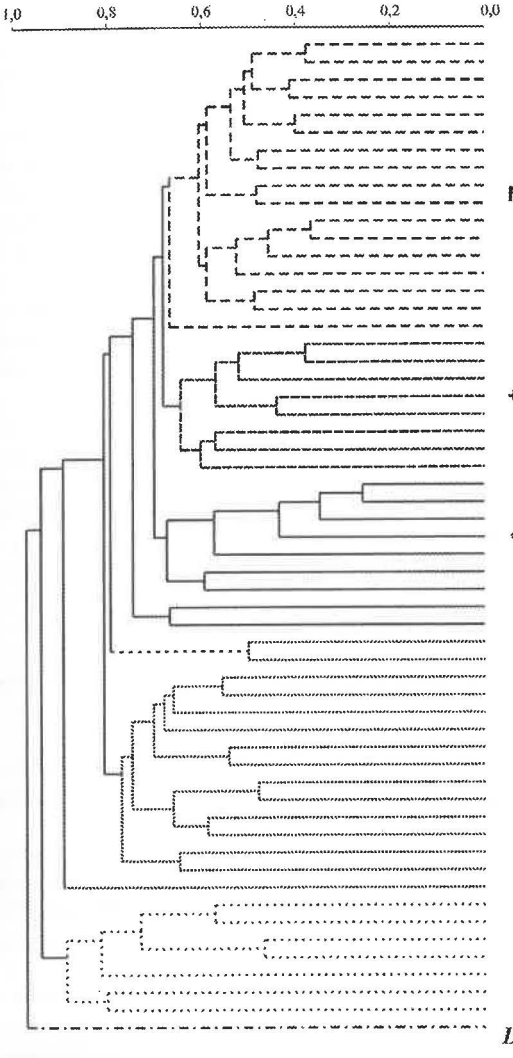
Keeping in mind the potentially harmful effects of cyanobacteria we propose a definition for harmful cyanobacterial blooms as follows:"A harmful cyanobacterial bloom is a seasonal dense phytoplankton growth, where more than 50% of the biomass comprises cyanobacteria, where the total chlorophyll value and the total phycocyanin value are each higher than 8 mg/m^3. (Tab. 1).

From this statement derives that harmful cyanobacterial blooms are mostly surface blooms and scums. Nevertheless they can present themselves in a less visible forms as metalimnetic blooms in stratified lakes or as dispersed dense blooms in eutrophic and hypertrophic water-bodies. In such blooms the concentration of cyanobacteria and their biologically active products is very high.

The accessory pigment phycocyanin is present in only few phytoplankton groups like *Cyanobacteria* and *Rhodophyta*. Additionally in our fresh waters the *Rhodophyta* species are exclusively benthic organisms. Phycocyanin is therefore in our opinion a better indicator for the presence of cyanobacterial blooms than chlorophyll. Since the contents of phycocyanin and chlorophyll are similar and, at the same time, extremely variable, we propose the same boundary values for the two photosynthetic pigments.

Our definition contains all the basic technical information necessary for easy identification, and is in conformity with the limits usually applied to potable and recreational waters. In Europe these limits are set at 10 mg m^{-3} of chlorophyll ***a***, which corresponds to ca. 10^7 cyanobacterial cells / l.

Field data on cyanobacterial blooms in Slovenia indicate that mesotrophic water bodies are potential and random sites of harmful cyanobacterial blooms, while the eutrophic and hypertrophic water bodies are sites with regular blooms with high microcystin production (Fig. 1). The metalimnetic blooms in natural lakes clearly show a different type of association demonstrating that the term "seemingly oligotrophic" is appropriate.



Legend:	OECD Value	(Chlorophyll mg/m³)	Cyanobacterial blooms
———	Hypertrophic	≥ 25	Frequent and regular
- - - - - -	Eutrophic	8 - 25	Frequent and regular
- - - - - -	Mesotrophic	2.5 - 8	Occasional
- · - · - · - L	Seemingly oligotrophic	≤ 2.5	Frequent and regular metalimnetic
·····	Oligotrophic	≤ 2.5	No
·····	Ultraoligotrophic	≤ 1	No

Figure1: Multivariate cluster analysis using a modified Bray-Curtis method (CLARKE & WARWICK 1990). Comparison of phytoplankton associations in surface water bodies of different trophic categories in Slovenia, based on chlorophyll contents of the water.

Slika 1: Multivariantna klasterska analiza po prilagojeni Bray-Curtisovi metodi (CLARKE & WARWICK 1990). Primerjava fitoplanktonskih združb v slovenskih površinskih vodnih telesih različnih trofičnih kategorij uvrščenih na podlagi vsebnosti klorofila.

Cyanobacterial bloom types in Slovenia

There are three basic types of cyanobacterial blooms in Slovenia which differ in the origin of nutrients:

*a.) **Planktonic blooms*** build up in eutrophic and hypereutrophic water bodies with nutrients evenly dispersed in the water, and where nutrient availability is influenced by diurnal stratification (all bloom forming species involved).

*b.) **Metalimnetic blooms*** build up in deeper mesotrophic and eutrophic reservoirs and in "seemingly" oligotrophic lakes where the nutrients become available as a consequence of seasonal stratification (predominantly filamentous species).

*c.) **Benthic blooms*** (cyanobacterial mats) build up in eutrophic and mezotrophic shallow water bodies, where the benthic cyanobacteria utilize nutrients from the sediment (*Oscillatoria princeps*).

In a temperate climate with seasonal changes, as in Slovenia, we are faced with stratification in all water bodies when the vertical mixing is weak. Therefore regard must also be paid to nutrient availability as a factor that triggers the beginning of cyanobacterial blooms (Reynolds 1984a). However, once the cyanobacterial bloom has started to form, light takes over as the major limiting factor in phytoplankton growth.

Basic cyanobacterial freshwater bloom configurations

Cyanobacterial blooms appear in different forms, depending on cyanobacterial abundance and on climatic and meteorological conditions.

– ***Dispersed blooms*** occur at the beginning of bloom formation and can appear secondarily as the consequence of vertical mixing due to high wind velocities (George & Edwards 1976). In such an environment the lower light conditions are mainly due to mutual shading of the plankton.

– ***Metalimnetic blooms*** occur in clear stratified lakes, where light penetrates beyond the depth of the epilimnion. Such blooms arise where opposing gradients of irradiance and nutrients are established due to the mobilisation of nutrients from the lake bottom (Ganf & Oliver 1982, Konopka 1989). In upper layers the light conditions are good and the position of cyanobacteria is due to their regulation of buoyancy (Reynolds & Walsby 1975),

– ***Surface blooms*** occur in calm weather and good insolation, when the speed of the wind is less than 2 – 3 m s^{-1}, resulting in low mixing rates (Webster & Hutchinson 1994). The light conditions below the bloom are bad, but are rescued by buoyancy regulation within the cyanobacterial population. Cyanobacteria alternatively migrate towards the surface in a constant exchange of cells and colonies at the water surface. Under favourable conditions such surface blooms may give the mistaken impression of a persistent bloom (Kromkamp & Walsby 1990).

– ***Scums*** originate from persistent blooms where there is a physical restraint on vertical movement (Ibelings & Mur 1992, Walsby 1994). Cyanobacterial scums prevent the penetration of light to deeper layers. The cells at the surface are often severly damaged by the high light intensities that can induce dehydration and cell senescence.

The most evident common characteristic of cyanobacterial blooms is the light environment, which ranges from low to very low and in extreme circumstances to even almost complete darkness. In several publications Mur emphasises the importance of light for cyanobacterial dominance. Results from competition experiments on the growth of *Scenedesmus* and *Oscillatoria* have shown that

cyanobacteria can reach higher growth rates than green algae only under extreme light limitation (*e.g.* MUR 1983).

Light limitation can be provoked by dispersed particles of different origin, or by mutual shading of phytoplankton. In shallow eutrophic water bodies there are different periodic progressions from one dominant phytoplankton assemblage to another (REYNOLDS 1980). These environments reduce the light to such an extent that only cyanobacteria remain competitive, giving rise in time to a massive population and out-competing other autotrophs.

With the appearance of cyanobacteria the light availability rapidly decreases. Gas vesicles present in buoyant cyanobacterial species induce additional horizontal light scattering that diminishes further light availability in deeper layers (WALSBY 1994).

Cyanobacterial blooms and microcystins in Slovenia

Our interpretations are based on results obtained from natural populations in Slovene water bodies, and on laboratory experiments with isolated cyanobacterial species and strains grown *in vitro* under controlled conditions. Our most frequent bloom forming cyanobacterial genera are *Microcystis*, *Anabaena*, *Aphanizomenon* and *Oscillatoria*.

Dispersed blooms are planktonic blooms, occurring in all eutrophic water bodies at the beginning of bloom formation or as a product of vertical mixing. They can be either toxic or non-toxic. The strains of cyanobacteria that are present at the beginning of bloom formation are in the majority of cases of non microcystin producing types.

Isolates of strains from different natural blooms have led to the identification of several non-producing and diverse producing strains of cyanobacteria. The isolation of a relatively high percentage of non-producing strains from toxic natural blooms can be explained by the fact that, for a successful isolation, single cells or filaments or small colonies are used. It has been namely demonstrated that larger colonies in most cases belong to producing strains (JUNGMANN & al. 1996). This observation leads to the assumption that producing strains are better adapted to bloom conditions, since they proliferate with faster dividing rates than non-producing strains, resulting in bigger colonies. Of course there are also several producing and non-producing strains that do not aggregate in colonies and proliferate in single cell configuration.

The evolution of a cyanobacterial bloom is a highly dynamic process in which a broad variety of strains are involved. The constant changes in the light environment in the bloom, due to the growth of cyanobacteria on the one hand and to meteorological and hydrological changes on the other, give different strains the opportunity to proliferate. With the aggravation of light conditions, strains that are better adapted predominate. It has long been known that *Microcystis* is non-toxic at the beginning of the growing season, but develops high toxicity during the first strong biomass increase (BENNDORF & HENNING 1989). Already in the sixties it was established that the possibility of a cyanobacterial bloom being toxic is over 50% (OLSON 1964). In our investigations this rises to over 80% (SEDMAK & al. 1994, SEDMAK & KOSI 1997a). The main difficulty in comparing such results from the literature lies in the poor definition of the bloom. Adopting our definition for cyanobacterial blooms and taking into consideration only the principal cosmopolite microcystin-producing genus *Microcystis*, the statement that cyanobacterial blooms evolve to toxic becomes a rule. We conclude that producing strains that prevail overwhelmingly in the bloom are better adapted to a low light environment. When we concentrate such dispersed blooms we can detect diverse microcystins that could originate also from different strains.

All filamentous and non-filamentous bloom-forming genera appear occasionally in the form of dispersed blooms.

Metalimnetic blooms are common in deeper stratified lakes and reservoirs. The main bloom forming species in Slovenia are filamentous *Aphanizomenon flos-aquae, Anabaena flos-aquae and Oscillatoria rubescens* (SEDMAK & KOSI 1997a, SEDMAK & KOSI 1991). They may be either producing or non-producing. We are concerned with Lake Bled, since it is the main centre of tourism in the region. The term "seemingly" oligotrophic is used because the productivity of the lake is normally low and the inflows are rich and permanent. The nutrients in the phase of summer stratification diffuse from the lake bottom and support metalimnetic blooms. *O. rubescens* grows almost every year when water stratification is established. In favourable meteorological and climatic conditions *Oscillatoria* migrates to the surface forming a surface bloom or even scum frequently covering almost the entire lake surface. Such blooms can persist on the surface even in January and can grow under the ice cover. In such cases we can normally detect microcystin-YR in bloom samples. It appears that the strain capable of MC-YR production is the best adapted to counter the lake environment. Lake Bled has two marked depressions which function as two independent sites of cyanobacterial growth. For this reason, unusual surface blooms can be observed as separate blooms of *O. rubescens* and *An. flos-aquae,* which subsequently merge in a unique mixed surface bloom. Blooms composed of equal parts of *O. rubescens* and *M. aeruginosa* have also been observed.

Surface blooms and scums prevail in summer and autumn in smaller eutrophic and hypertrophic water bodies such as reservoirs, fishponds and abandoned gravel pits. The main species are *Microcystis aeruginosa, Microcystis wesenbergii* and *An. flos-aquae* (Sedmak & Kosi 1997b). *Microcystis* species are almost always toxic. Occasionally there are also blooms of *Aphanizomenon flos-aquae, Oscillatoria limnetica* and *Oscillatoria agardhii,* which till now were all identified as non-producing. Mixed blooms are also common. *M. aeruginosa* appears frequently together with *M. wesenbergii* or *An. flos-aquae*.

Benthic cyanobacterial species like *O. princeps* can also rise to the surface and form surface blooms in the case of high proliferation rates. So far we have not been able to detect productive strains of this species. However there are some cases when microcystins have been produced by benthic cyanobacteria (MEZ et al. 1997).

All blooms have at least two things in common, high fluctuations in oxygen content and high light limitation. Additionally, the *Microcystis* blooms and scums are, to a large extent, associated with microcystin production. These blooms in a similar environment in North-eastern Slovenia all end with an almost identical pattern of production of microcystins-RR and –LR (SEDMAK & KOSI 1977a). Again we can assume that these productive strains are the best adapted to take over the highly eutrophic water bodies in the specific environment of the region.

A concise survey of microcystin production and their possible role

Today it is perfectly clear that the ability of a strain to produce microcystins depends on its possession of the necessary genes (MEIßNER et al.1996; DITTMANN et al. 1997). Thus there are strains able to produce microcystins to different extents and others that are not able to produce them at all. Meanwhile, the amount of production is dependent principally on ecological conditions (*e.g.* WATANABE & OISHI 1985). So it is obvious that the success of a particular strain depends on its adequacy under given conditions. There is great intraspecific biodiversity in the production of those biologically active substances that benefit the producing organisms, which is also the case in microcystin synthesis (NEILAN et al. 1999). So in the space of time from the origin of a bloom to its senescence, we have a

series of physiologically diverse cyanobacteria that can be successful to different degrees in various conditions, even in the framework of the same species. The possibility of natural genetic transformation in bloom conditions is very low due to the short time span of the bloom and relatively low dividing rates of cyanobacteria.

So far it seems that there is no single factor responsible for the variation in toxicity of cyanobacteria. From various data *in vitro* as well as from data obtained from natural blooms, it is evident that the differences in microcystin production between strains are very large, microcystin contents ranging from zero to 1.5% of cyanobacterial biomass according to JUNGMANN and co-workers (1996) and 1.84% microcystins /dry weight according to UTKILEN and GJØLME (1992). In our analyses total microcystin content can reach 2% of cyanobacterial dry weight (calculated value 0.64 pg/cell) in natural populations (SEDMAK & KOSI 1997a). On the other hand, the chlorophyll ***a*** content of *M. aeruginosa* is on average 1.5% of cell dry weight (0.26 – 0.43 pg/cell) (REYNOLDS 1984b).

Such huge microcystin production, comparable to the content of the indispensable chlorophyll ***a***, emphasizes the ecological importance of these substances for the producing cyanobacteria. The most recent findings indicate that microcystin synthesis proceeds via a multienzyme complex consisting of both peptide synthetase and polyketide modules (KAEBERNICK & NEILAN 2001). In such a synthesis significant cellular energy is required. The role of microcystin should be correspondingly great.

As already mentioned, cyanobacteria were among the first organisms to inhabit our planet. It is more probable that their evolution involved biologically active substances that would aid them in their adaptive capabilities rather than harm rivals that did not even exist. From a quick survey of scientific data we can summarize that there is no evolutionarily adaptive value for cyanobacteria to kill land animals and fish (JUNGMANN et al. 1996). It can be expected that cyanobacteria and their products interact primarily with organisms in the same environment. Microcystins are not likely defence substances against grazers, since experiments have demonstrated that they are toxic to zooplankton only at very high concentrations (DE MOTT et al. 1991) and that there are other, more effective substances against them isolated from cyanobacteria (*e.g.* NIZAN et al. 1986). Therefore according to *Occam's razor* (WILLIAM OCKHAM 1285 – 1349) whereby »unnecessary assumptions should be abandoned«, the assumption that microcystins are defence substances is, in our opinion, unnecessary.

We have proposed that there is no environmental factor capable of converting a non-producing strain into a producing strain (SEDMAK & KOSI 1998a, SEDMAK & KOSI 1998b), but that there must be an ecological factor that augments the growth of microcystin producing cyanobacteria in order to prevail over other species and strains. In the last decade, research has been focused on environmental factors that could trigger microcystin production, rather than looking at cyanobacterial species as complex mixtures of different strains with diverse adaptive values.

Light availability and microcystin production

Work on microcystin production has been concentrated on the study of specific producing strains of cyanobacteria, in order to find optimal conditions where they proliferate and produce and with the aim of understanding how they would behave in bloom conditions. However, in specific bloom conditions, they may represent changeable proportion of the cyanobacterial biomass. In nature it is the environment that gives the opportunity to the fittest. During the evolution of the bloom there is a constant change of conditions. Specific strains are favoured and, with their proliferation, there is, in turn, an additional change in environmental conditions that offers the opportunity to another better adapted strain to propagate. With the growth of the bloom, light conditions become worse and only strains capable of proliferation under such extreme environments can prevail. In our opinion, microcystin producing strains are better adapted to low light conditions than non-producing strains (SEDMAK

2001). This explains why the blooms become more toxic with the increase in cyanobacterial biomass. It also explains the poor permeability of cells to microcystins, which are primarily designed to influence their own physiology.

Orr and Jones (1998) have shown that the highest microcystin concentrations are produced under conditions optimal for cell growth. There is a high probability therefore that under optimal conditions there will be an optimal production of all the microcystin variants that a strain is capable of producing. These optimal conditions for producing cyanobacteria coincide with relatively low light conditions. It is necessary therefore to be able to estimate the availability of light in eutrophic water bodies with abundant phytoplankton growth. The optimal light intensities for producing *M. aeruginosa* strains have been estimated as being less than 40 microeinsteins m^{-2} s^{-1} (Utkilen & Gjølme 1992). Similar values have been reported for other microcystin producing cyanobacteria (Rapala et al. 1997). Such intensities are normally found in cyanobacterial blooms at a depth of about 1 m (Utkilen & Gjølme 1992). From these data it is evident that producing cyanobacteria are adapted to the low light conditions characteristic of an already existing bloom. Cyanobacteria that start such an environment usually belong to the non-producing or poorly producing strains. Microcystin production is energy consuming and becomes an advantage only in an adequate environment. We have found a strong positive correlation between microcystin production and cyanobacterial cell concentration in the bloom, suggesting that producing strains are capable of more dense bloom and scum formation and of survival in consequent low light environment (Fig 2).

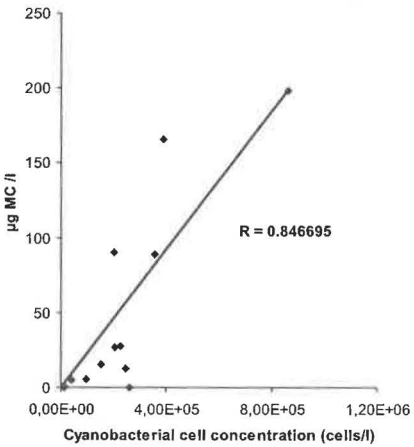


Figure 2: The relationship the cumulative values of produced microcystins (ΣMC) in the bloom and the cyanobacterial cell concentration.
Slika 2: Razmerje med vsebnostjo vseh mikrocistinov (ΣMC) in koncentracijo cijanobakterijskih celic v cvetu.

There have been a few attempts to find a linkage between photosynthesis and microcystin production. Microcystins have been found primarily in the thylakoid and nucleoid regions (Shi et al. 1995) and it is believed that the ADDA moiety of the toxin may bind to the thylakoid. This association with the photosynthetic apparatus of cyanobacterial cells may indicate a function in the light harvesting and chromatic adaptation mechanisms exhibited by these organisms (Orr & Jones 1998) Light dependent processes are essential to both prokaryotic cyanobacteria and eukaryotic algae, which compete in the same habitat. Thus the production of biologically active substances that gives an advantage in this crucial area is clearly supported in the process of evolution.

Can microcystins influence other phytoplankton species in the environment?

Despite the established opinion that microcystins are generally not cell-permeable, except the hepatocytes, which have a specific uptake system, we have demonstrated that they can influence the growth of different phytoplankton species in culture even at low concentrations (10^{-7} M) (SEDMAK & KOSI 1998a, SEDMAK & KOSI 1998b). Recent investigations have confirmed the possibility of non-specific translocation of oligopeptides (to undecamer) across plasma membranes (OELHKE et al. 1997). Microcystins are heptapeptides and as such they can pass the cell membrane. Whether the release of microcystins is due to cell death or to cell leakiness, it remains a fact that microcystins can be detected in the environment during cell proliferation (RAPALA et al. 1997). In such a way, non-producing strains also may persist longer in the bloom, preserving a bigger biodiversity of strains. *In vitro* experiments have namely confirmed that non-producing strains exposed to microcystins achieve higher proliferation rates under low light conditions than otherwise (SEDMAK & KOSI 1998a, SEDMAK & KOSI 1998b). Such diverse cyanobacterial association can dominate for a longer period in the continuously changing water environment. In such an unpredictable situation, the role of microcystins is also to preserve the variability of strains. The cyanobacterial bloom is functioning as a superorganism where different strains take over in conformity with temporary conditions. This assumption is supported by the fact that we can isolate non-producing strains, irrespective of how dense and toxic the monospecific cyanobacterial bloom or scum may be.

The presence of microcystins can also influence the growth of other phytoplanktonts in the bloom. The diversity in the blooms is namely low. We have found a correlation between microcystin production and the presence of phytoplankton species in toxic cyanobacterial blooms (Fig.3). We suggest that there is a combined effect of light limitation together with microcystin influence on susceptible phytoplankton species.

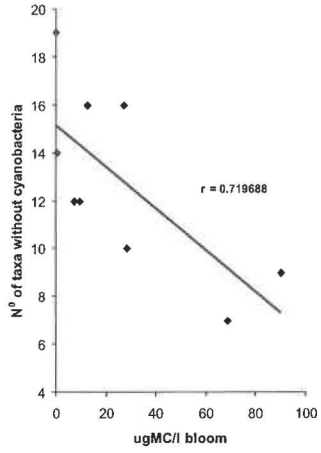


Figure 3. The relationship between microcystin production in the bloom and phytoplankton diversity.
Slika 3: Razmerje med koncentracijo mikrocistinov v cvetu in biodiverziteto fitoplanktona.

Cyanobacteria can control their buoyancy, but in old and very dense blooms the competition for carbon dioxide can depress photosynthesis to such a degree, that the turgor pressure in the cell can no longer rise and the gas vesicles are not able to collapse. Such a bloom is thus trapped at the water surface (*e.g.* WALSBY 1994). Exposure to direct sunlight, with photooxidation of the photosynthetic pigments, leads to the death and disintegration of cyanobacteria. In this situation, the moribund and lysed cells release massive amounts of microcystins into the water. They can stimulate the growth of other phytoplankters (*i.e. Scenedesmus* spp.), that tolerate the presence of microcystins, giving rise to another, this time green algae, bloom. Frequently, after the collapse of a cyanobacterial bloom, green algae become dominant (LIN 1972). In our opinion the disintegration of a hepatotoxic bloom accelerates the proliferation of defined tolerant genera (SEDMAK & KOSI 1998a, SEDMAK & KOSI 1998b), while several other susceptible taxa can be excluded from the environment.

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Ventilatory and anthropometric variables in healthy female students from the University of Ljubljana

Ventilacijski in antropometrični parametri zdravih študentk Univerze v Ljubljani

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Abstract. Forced expiratory vital capacity (FEVC) and forced expiratory volume in the first second (FEV_1) were measured in healthy, non-smoking female students from the University of Ljubljana. The sample included 86 students, none active in sports, ranging in age from 18-23 years. The results were analysed in terms of anthropometric variables, and regression equations were derived. Comparisons were made with Europaean Respiratory Society (ERS) prediction equations derived from non-Slovenian populations, commonly used in our medical practice. The ERS and our equations predict significantly different values for FEVC and insignificantly for FEV_1. According to comparisons of r^2 and SD of residuals, we presumed that newly derived equations can be better predictors of ventilatory parameters for Slovene female students population.

Keywords: students, female, physiology, ventilation, spirometry, anthropometry, vital capacity, forced expiratory volume.

Izvleček. Pri zdravih študentkah nekadilkah, ki se s športom ne ukvarjajo aktivno, smo merili forsirano ekspiratorno vitalno kapaciteto (FEVC) in forsirani ekspiratorni volumen 1 (FEV_1). Vzorec je obsegal 86 študentk ljubljanske universe, starih od 18 do 23 let. Iz izmerjenih antropometričnih parametrov smo izpeljali dve regresijski enačbi za izračun FEVC in FEV_1. Rezultate smo primerjali z rezultati Evropskega respiratornega združenja (ERS), ki se običajno uporabljajo v medicinski praksi. Izmerjene vrednosti FEVC značilno in vrednosti FEV_1 neznačilno presegajo predvidene evropske standarde. Smatramo, da so za slovensko žensko populacijo naše predikcijske enačbe primernejše, zato priporočamo uporabo lastnih standardov.

Ključne besede: študentke, fiziologija, spirometrija, antropometrija, vitalna kapaciteta, forsirani ekspiratorni volumen.

Introduction

Lung volumes and spirometric tests are routinely used to evaluate the normality of respiratory functions. This is achieved by comparing laboratory measured values with those predicted from multiple regression equations that have been developed on a reference sample; the equations frequently use age and body height as independent or predictor variables (WITHERS & al. 1989a, BECKLAKE & al. 1991, QUANJER & al. 1993). There are often very significant variations in predicted values for lung function indices. Such variation reflects, among other considerations (BECKLAKE & al. 1991, ZUSKIN & al. 1996), differences in the population studied, equipment used to measure lung functions and methodology employed. As there are, at present, no references values for any adult Slovenian population, the results of spirometric testing have been mostly evaluated using reference values developed in other populations of Europe. Many studies have found that using reference values developed in other populations of Europe and North America in practice are inadequate for some lung function parameters (WITHERS & al. 1989a,b, SMOLEJ-NARANČIĆ & al. 1991, BRÄNDLI & al. 1996). The objectives of this investigation were, therefore, to define the correlations between spirometric values and measured anthropometric variables and to provide reliable standards in the form of prediction equations.

Subjects and methods

The study included 86 non-smoking female students, not participating in active sports. Only students without a history of respiratory illness or not showing its symptoms before or during the study and without chest wall deformity were included. We used a Standardized Medical Research Council questionnaire (QUANJER & al. 1993) to screen the subjects on the basis of having:

1. never suffered from asthma, chronic bronchitis, pneumonia, pulmonary tuberculosis, pleurisy
2. never had a persistent cough and/or phlegm for at least 3 months in a year
3. never had chest surgery and/or a major chest injury
4. never lived in a heavily polluted environment

The students came from different parts of Slovenia, but the majority of them were from the city of Ljubljana. On average, they were 20.2 years old. All the measurements were performed in the autumn of 1996 between 8am and 1pm, by the same technician.

Spirometry

Pulmonary function tests consisted of measuring forced expiratory vital capacity (FEVC) and its subdivision, forced expiratory volume in the first second (FEV_1). The results were recorded according to recommendations of the European Respiratory Society (QUANJER & al. 1993). Tests were conducted at an altitude of 350m above mean sea level, using a Spiro 323 spirometer (P.K. Morgan Instruments, Inc.). The spirometer was calibrated on a daily basis, using a precision syringe. Barometric pressure, water vapour pressure and ambient temperature were recorded daily. Recorded volumes were expressed as body temperature and pressure saturated with water vapour (BTPS). All the tests were performed with the subjects in a sitting possition and a nose-clip was used. Prior to their commencement, a careful demonstration of the tests was given to the subjects. FEVC measurements were repeated until three FEVC values were obtained that varied no more than 100 ml or 5%, and the highest values accepted. The data in Tab. 1 demonstrate a high degree of test retest reliability for ventilatory and anthropometric

variables. For predicting lung volumes of the non-smoking adult woman, we used the equations recommended by the European Respiratory Society (QUANJER & al. 1993) that are commonly used in Slovenian medical practice (see Tab. 4). They were derived from studies carried out on adult subjects (18-70 years) of European descent, who were non-smokers and without (previous) disease which could compromise their ventilatory function.

Table 1: Test-retest reliability statistic for lung volumes and selected anthropometric variables
Tabela 1: Napaka meritve izmerjenih ventilacijskih parametrov in antropometričnih mer

Variable	Mean of the first measurements	Mean of the second measurements	$\overline{\lvert d \rvert}$	SEE	r
FEVC (l)	4.14	4.11	0.11	0.09	0.97
FEV_1 (l)	3.58	3.59	0.07	0.06	0.98
Weight (kg)	60.6	60.8	0.21	0.37	0.99
Height (cm)	167.1	167.2	0.12	0.51	0.98
Sitting height (cm)	88.1	87.8	0.29	0.45	0.97
Arm span (cm)	165.6	165.9	0.26	0.57	0.99
Shoulder width (cm)	37.2	37.2	0.03	0.38	0.93
Width of chest (cm)	26.1	26.1	0.02	0.45	0.94
Depth of chest (cm)	16.1	16.3	0.27	0.46	0.90
Chest circumference at TLC (cm)	90.1	90.5	0.38	1.09	0.93
Chest circumference at FRC (cm)	84.5	84.6	0.11	1.27	0.94
Chest circumference at RV (cm)	87.0	86.6	0.42	1.20	0.94
Thickness of triceps skin-fold (mm)	14.2	13.9	0.37	0.62	0.97
Thickness of subscapular skin-fold (mm)	11.2	10.9	0.22	0.67	0.98
Thickness of midaxillary skin-fold (mm)	10.1	10.4	0.33	1.14	0.96
Thickness of suprailiac skin-fold (mm)	12.5	12.2	0.26	1.21	0.97
Thickness of medial calf skin-fold (mm)	16.1	15.9	0.29	0.94	0.98

FRC: functional residual capacity
RV: residual volume

$\overline{\lvert d \rvert}$: mean of the absolute differences

SEE: standard error of estimate
r: the coefficient of reliability

Anthropometry

Anthropometric variables were measured according to the requirements of the International Biological Programme (WEINER & LOURIE 1969), using standard anthropometric equipment. Stature was measured with an Siber-Hegner anthropometer to an accuracy of 0.1 cm. Body weight was measured on digital Soehnle scales to an accuracy of 0.1 kg. Skin-fold thicknesses were taken with a John Bull calliper, under a constant pressure of 10 gmm^{-2}. The same instruments were used throughout the study.

Statistical analyses

All the statistical analyses were performed using a pocket Statistica 4.3 Statsoft program. After checking variables for normality and linearity of distribution, bivariate correlations between measured parameters were calculated. Forward stepwise multiple regression analyses were made to derive equations for predicting FEVC and FEV_1 values from the best weighted combination (with significance $p<0.01$) of anthropometric or other predictor variables. All anthropometric parameters were included

in the regression. Equations were validated with a t-test for testing for differences between correlation coefficients (POLLARD 1977). We used two correlation coefficients: r_1 (between FEVC or FEV_1 values recorded and FEVC or FEV_1 values predicted with ERS equations) and r_2 (between FEVC or FEV_1 values recorded and FEVC or FEV_1 values predicted according to our equations). If there were any statistically significant ($p<0.05$) differences between r_1 and r_2 we presumed that the commonly used and newly derived equations were predicting significantly different values for ventilatory parameters. The choice of the best equation was based on an analyses of residuals, a comparison of the residual standard deviation, i.e. standard error of estimate (SEE) and comparison of the proportion of total variance in lung function explained by the model (r^2).

Results

The mean values and standard deviations for lung volumes, age and selected anthropometric variables are listed in Tab. 2. The mean FEVC was 4.2 liters and FEV_1 was 3.7 liters. The correlation matrix of measured variables, shown in Tab. 3, demonstrate that the measurements of the chest correlated significantly with FEVC and FEV_1, but weaker than height, sitting height and arm span. The thicknesses of skin-folds were negatively correlated with ventilatory parameters, but only for medial calf skin-fold the correlation was statistically significant.

Table 2: The mean values and standard deviations for measured parameters
Tabela 2: Povprečja in standardne devijacije izmerjenih parametrov
TLC: total lung capacity

Variable	Mean	SD
FEVC(l)	4.2	0.46
FEV_1(l)	3.7	0.40
Age (years)	20.2	1.50
Weight (kg)	60.7	7.98
Height (cm)	167.0	5.78
Sitting height (cm)	87.8	3.05
Arm span (cm)	165.2	7.00
Shoulder width (cm)	36.0	1.76
Width of chest (cm)	25.9	1.31
Depth of chest (cm)	15.9	1.22
Chest circumference at TLC (cm)	89.6	5.05
Chest circumference at FRC (cm)	86.3	5.28
Chest circumference at RV (cm)	84.3	5.36
Thickness of triceps skin-fold (mm)	15.7	4.31
Thickness of subscapular skin-fold (mm)	11.2	3.44
Thickness of midaxillary skin-fold (mm)	10.8	4.62
Thickness of suprailiac skin-fold (mm)	13.3	5.41
Thickness of medial calf skin-fold (mm)	17.0	6.25

FRC: functional residual capacity
RV: residual volume

Table 3: The correlation matrix of measured variables

Tabela 3: Korelacije izmerjenih parametrov

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 FEVC(l)		0.76*	0.20	0.27*	0.64*	0.51*	0.61*	0.50*	0.31*	0.37*	0.41*	0.35*	0.30*	-0.15	-0.11	-0.15	-0.04	-0.27*
2 FEV_1(l)			0.13	0.17	0.62*	0.49*	0.60*	0.31*	0.22*	0.29*	0.29*	0.25*	0.24*	-0.20	-0.15	-0.16	-0.11	-0.30*
3 Age (years)				-0.12	-0.18	-0.33	-0.14	-0.13	-0.18	0.13	0.05	-0.05	-0.00	-0.08	-0.07	-0.01	-0.04	-0.16
4 Weight (kg)					0.42*	0.42*	0.43*	0.38*	0.54*	0.73*	0.81*	0.83*	0.82*	0.46*	0.30	0.37*	0.47*	0.15
5 Height (cm)						0.90*	0.90*	0.60*	0.67*	0.12	0.56*	0.53*	0.43*	-0.11	-0.30	-0.19	-0.17	-0.41*
6 Sitting height (cm)							0.76*	0.48*	0.72*	0.14	0.52*	0.56*	0.47*	0.00	-0.12	-0.12	-0.16	-0.16
7 Arm span (cm)								0.63*	0.68*	0.14	0.57*	0.49*	0.39*	-0.09	-0.34	-0.14	-0.15	-0.39*
8 Shoulder width (cm)									0.44*	0.13	0.45*	0.38*	0.30	-0.12	-0.21	-0.14	-0.01	-0.33
9 Width of chest (cm)										0.34	0.67*	0.68*	0.63*	0.05	-0.14	-0.04	-0.10	-0.15
10 Depth of chest (cm)											0.65*	0.69*	0.68*	0.28	0.18	0.34	0.27	0.10
11 Chest circumference at TLC (cm)												0.95*	0.93*	0.10	0.05	0.21	0.12	-0.14
12 Chest circumference at FRC (cm)													0.98*	0.17	0.18	0.24	0.14	-0.06
13 Chest circumference at RV (cm)														0.22	0.27	0.30	0.18	0.02
14 Thickness of triceps skin-fold (mm)															0.64*	0.55*	0.51*	0.68*
15 Thickness of subscapular skin-fold (mm)																0.71*	0.59*	0.50*
16 Thickness of midaxillary skin-fold (mm)																	0.62*	0.32
17 Thickness of suprailiac skin-fold (mm)																		0.30
18 Thickness of medial calf skin-fold (mm)																		

$p<0.05$

TLC: total lung capacity

FRC: functional residual capacity

RV: residual volume

The regressions of lung functions parameters on anthropometric variables contributing significantly to the description of lung volumes and the ERS equations, are given in Tab. 4. The evaluated prediction equation for FEVC included the chest circumference at total lung capacity, the thickness of midaxillary skin-fold, of medial calf skin-fold and of triceps skin-fold. The prediction equation for FEV_1 is based on the chest circumference at residual volume, the thickness of midaxillary skinfold and on shoulder width. As shown, the observed values exceeded those predicted by ERS equations by, on average, 0.20 liters for FEVC and 0.22 liters for FEV_1.

Table 4: Regression equations for FEVC and FEV_1, evaluated on a sample of female students, and ERS equations, the percent of explained variance and the standard error of estimation for FEVC in FEV1

Tabela 4: Regresijski enačbi za izračun FEVC in FEV_1 izpeljani iz populacije študentk in ERS enačbi, prikaz deleža pojasnjene variabilnosti v vzorcu in standardne napake pri oceni dejanske FEVC in FEV1

Lung volume	Evaluated regression equations	r^2 %	Diff.	SD	SEE
FEVC(l)	0.054CCT – 0.042TMS – 0.029TCS + 0.020TTS	47.6	0.00	0.336	0.237
FEV_1(l)	0.040CCR – 0.045TMS + 0.024SW	24.5	0.00	0.348	0.246
	ERS equations (QUANJER & al. 1993)				
FEVC(l)	4.43H – 0.026A – 2.89	33.5	0.20	0.367	0.259
FEV_1(l)	3.95H – 0.025A –2.60	34.6	0.22	0.322	0.228

CCT: chest circumference at total lung capacity (cm)
TMS: thickness of midaxillary skin-fold (mm)
TCS: thickness of medial calf skin-fold (mm)
TTS: thickness of triceps skin-fold (mm)
CCR: chest circumference at residual volume (cm)
SW: shoulder width (cm)
H: body height (cm)
A: age (years)
r^2:% of explained variance
Diff: difference between mean observed and mean predicted
SD: standard deviation of residuals
SEE: standard error of estimate FEVC and FEV_1

The correlation coefficients, listed in Tab. 5, show that ERS equations and our regression equations predict statistically significant different values for FEVC and insignificantly for FEV_1.

Table 5: Testing for difference between observed and predicted FEVC and FEV_1
Tabela 5: Razlika med dejanskimi in predvidenimi vrednostmi FEVC in FEV_1

	FEVC(l)	t	FEV_1(l)	t
r_1	0.58	2.49*	0.59	0.90
r_2	0.68		0.49	

$p<0.05$

r_1 = correlation coefficient between observed and by ERS equations predicted volumes

r_2 = correlation coefficient between observed volumes and volumes predicting according to our equations

Discussion and conclusions

The results shown are valid for a healthy, non-smoking population of female students from the University in Ljubljana. Prediction equations for FEVC and FEV_1 frequently use age and body height (WITHERS & al. 1989a, BECKLAKE & al. 1991, QUANJER & al. 1993) as predictor variables. However, due to the narrow age range in this group (18-23 years), the correlation of lung functions with age is low and statistically insignificant (Tab. 3). A sample population with a greater age range would show a significant, negative correlation between age and the measured ventilatory parameters, as it is known (KNUDSON & al. 1977) that in an ageing adult population, the values of lung functions fall, principally due to a loss in elasticity of the lung and thoracic walls.

The uniformity of body height and age, is the cause of them not demonstrating good prediction of lung functions. Correlations between lung functions and the body length measurements such as body and sitting height, were higher and statistically significant. It is know that FEVC and FEV_1 values highly depends on stature (COTES & al. 1979, MALIK & al. 1972).

Thorax dimensions show also positive and significant correlations with FEVC and FEV_1. SING & BHASIN (1983) allege that such correlations are more evident in people who live at high altitude. The quantity of fatty tissue under the skin correlates negatively with ventilatory parameters (COLLINS & al. 1995, LAZARUS & al. 1997) as shown in Tab. 3. The correlation between body weight and lung functions is characteristically positive for which the cause is most likely the muscle-bone body component (BRODAR 1981) that in early adulthood, show a higher positive correlation with body weight. In ageing populations of constant body weight, the proportion between body fat and lean body weight changes in favour of the fat component. Negative correlations between increased body weight at the expense of acquired fat and lung functions are seen (CHEN & al. 1993, CHINN & al. 1996, WANG & al. 1996).

ERS prediction equations commonly used in Slovenia, on average underestimate actual ventilatory values, FEVC and FEV_1 as shown in Tab. 4. The independent variables which contributed significantly to the description of a dependent variable FEVC in the derived regression equation are chest circumference at total lung capacity (TLC), thickness of midaxillary, medial calf and triceps skin-folds. No improvement of r^2 or SEE was obtained in regression analyses by the addition of other variables.

Using a t-test, we determined that the commonly used and our regression equation predict significant different values of FEVC, that in practice would mean a significantly different interpretation of ventilatory functions (Tab. 5). We believe, that the newly formed regression equation is a better

predictor of FEVC in the female student population in Slovenia.

FEV_1 in the derived regression equation can be predicted from the chest circumference at residual volume (RV), shoulder width and thickness of triceps skin-fold. From Tab. 5, it is evident that the predictor of FEV_1 is not significantly different with the commonly used and newly derived regression equation. We believe that our equation can be a predictor of FEV_1 in a population of female students, but are unable to say that it is better than the ERS equation.

We conclude with an observation that measurements of FEVC and FEV_1 on average, significantly exceed the values of those predicted with the ERS equations. This is the result of the difference between the Slovenian population and the population from which are derived the ERS equations that we use in practice. To provide reliable standards, we must derive regression equations on a larger and more heterogeneous sample.

Povzetek

Ventilacijske funkcije vrednotimo s spirometričnimi metodami, tako, da izmerjene vrednosti ventilacijskih parametrov primerjamo z referenčnimi. Referenčne vrednosti navadno izračunamo z regresijskimi enačbami, ki so spolno specifične in kot neodvisne spremenljivke vključujejo starost in telesno višino. V Sloveniji nimamo svojih standardov, zato ventilacijske funkcije vrednotimo z regresijskimi enačbami izpeljanimi na drugih evropskih populacijah.

Namen tega dela je bil izpeljati regresijske enačbe za izračun forsirane ekspiratorne vitalne kapacitete (FEVC) in forsiranega ekspiratornega volumna 1 (FEV_1) iz antropometričnih mer zdravih slovenskih študentk, ki se s športom aktivno ne ukvarjajo.

Antropometrične meritve smo izvajali po mednarodnih priporočilih - IPB (WEINER & LOURIE 1969), spirometrijo pa v skladu z zahtevami Evropskega respiratornega združenja – ERS (Quanjer & al. 1993).

Povprečna FEVC slovenskih študentk znaša 4,3 litra, povprečni FEV_1 pa 3,7 litra (Tab.2). Mere prsnega koša so značilno pozitivno povezane z vrednostima ventilacijskih funkcij, vendar šibkeje kot dolžinske mere telesa. Debelina kožnih gub značilno negativno korelira z FEVC in FEV_1 (Tab.3). Izmerjena ventilacijska parametra v povprečju presegata vrednosti izračunane z ERS predikcijskima enačbama, in sicer za 0.20 litra v primeru FEVC in 0.22 litra za FEV_1. Izpeljali smo regresijsko enačbo za izračun FEVC iz mere obsega prsnega koša ob največjem vdihu ter debeline kožne gube na tricepsu, goleni in midaksilarne kožne gube. Regresijska enačba za izračun FEV_1 vključuje obseg prsnega koša ob izdihu do rezidualnega volumna, širino ramen in debelino midaksilarne kožne gube (Tab.4). ERS regresijska enačba predvideva značilno drugačne vrednosti FEVC kot naša regresijska enačba, kar pa ne velja za FEV_1 (Tab.5). ERS regresijske enačbe podcenjujejo dejanske vrednosti FEVC in FEV_1 slovenskih študentk. Navedeno lahko privede do napačnega vrednotenja pljučnih funkcij v tej skupini. Menimo, da izpeljana regresijska enačba za izračun FEVC predvideva vrednosti, ki so bližje dejanskim kot ERS enačba. Regresijska enačba za FEV_1 pa vrednosti, ki so najmanj toliko podobne dejanskim kot izračuni z ERS enačbo.

Zaključujemo, da potrebujemo lastne standarde, s katerimi bi lahko pravilno vrednotili ventilacijske funkcije slovenskih študentk.

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NAVODILA AVTORJEM

1. Vrste prispevkov

a) ZNANSTVENI ČLANEK je celovit opis originalne raziskave in vključuje teoretični pregled tematike, podrobno predstavljene rezultate z diskusijo in sklepe ter literaturni pregled: shema IMRAD (Introduction, Methods, Results And Discussion). Dolžina članka, vključno s tabelami, grafi in slikami, ne sme presegati 15 strani; razmak med vrsticami je dvojen. Recenzirata ga dva recenzenta.

b) PREGLEDNI ČLANEK objavi revija po posvetu uredniškega odbora z avtorjem. Število strani je lahko večje od 15.

c) KRATKA NOTICA je originalni prispevek z različnih bioloških področij (sistematike, biokemije. genetike, mikrobiologije, ekologije itd.), ki ne vsebuje podrobnega teoretičnega pregleda. Njen namen je seznaniti bralca s preliminarnimi ali delnimi rezultati raziskave. Dolžina na sme presegati 5 strani. Recenzira ga en recenzent.

d) KONGRESNA VEST seznanja bralce z vsebinami in sklepi pomembnih kongresov in posvetovanj doma in v tujini.

e) DRUŠTVENA VEST poroča o delovanju slovenskih bioloških društev.

2. Originalnost prispevka

Članek, objavljen v reviji Acta Biologica Slovenica, ne sme biti predhodno objavljen v drugih revijah ali kongresnih knjigah.

3. Jezik

Teksti naj bodo pisani v angleškem jeziku, izjemoma v slovenskem, če je tematika zelo lokalna. Kongresne in društvene vesti so praviloma v slovenskem jeziku.

4. Naslov prispevka

Naslov (v slovenskem in angleškem jeziku) mora biti kratek, informativen in razumljiv. Za naslovom sledijo imena avtorjev in njihovi polni naslovi (če je mogoče, tudi štev. faxa in e-mail).

5. Izvleček - Abstract

Podati mora jedrnato informacijo o namenu, uporabljenih metodah, dobljenih rezultatih in zaključkih. Primerna dolžina za znanstveni članek naj bo približno 250 besed, za kratko notico pa 100 besed.

6. Ključne besede - Keywords

Število naj ne presega 10 besed; predstavljati morajo področje raziskave, obravnavane v članku. Člankom v slovenskem jeziku morajo avtorji dodati ključne besede v angleškem jeziku.

7. Uvod

Nanašati se mora le na tematiko, ki je predstavljena v članku ali kratki notici.

8. Slike in tabele

Tabele in slike (grafi, dendrogrami, risbe, fotografije idr.) naj v članku ne presegajo števila 10, v članku naj bo njihovo mesto nedvoumno označeno. Ves slikovni material naj bo oddan kot fizični original (fotografija ali slika). Tabele in legende naj bodo tipkane na posebnih listih (v tabelah naj bodo le vodoravne črte). Naslove tabel pišemo nad njimi, naslove slik in fotografij pod njimi. Naslovi tabel in slik ter legenda so v slovenskem in angleškem jeziku. Pri citiranju tabel in slik v besedilu uporabljamo okrajšave (npr. Tab. 1 ali Tabs. 1-2, Fig. 1 ali Figs. 1-2; Tab. 1 in Sl. 1).

9. Zaključki

Članek končamo s povzetkom glavnih ugotovitev, ki jih lahko zapišemo tudi po točkah.

10. Povzetek - Summary

Članek, ki je pisan v slovenskem jeziku, mora vsebovati še obširnejši angleški povzetek. Velja tudi obratno.

11. Literatura

Uporabljene literaturne vire citiramo med tekstom. Če citiramo enega avtorja, pišemo ALLAN (1995) ali (ALLAN 1995), če sta dva avtorja (TRINAJSTIĆ & FRANJIĆ 1994), če je več avtorjev (PULLIN & al. 1995). Kadar navajamo citat iz večih del hkrati, pišemo (HONSIG-ERLENBURG & al. 1992, WARD 1994a, ALLAN 1995, PULLIN & al. 1995). V primeru, če citiramo več del istega avtorja, objavljenih v enem letu, posamezno delo označimo s črkami a, b, c itd. (WARD 1994a,b). Če navajamo dobesedni citat, označimo dodatno še strani: TOMAN (1992: 5) ali (TOMAN 1992: 5-6). Literaturo uredimo po abecednem redu, začnemo s priimkom prvega avtorja, sledi leto izdaje in naslov članka, mednarodna kratica za revijo (časopis), volumen poudarjeno, številka v oklepaju in strani. Npr.:

HONSIG-ERLENBURG W., K. KRAINER, P. MILDNER & C. WIESERR 1992: Zur Flora und Fauna des Webersees. Carinthia II **182/102** (1): 159-173.

TRINAJSTIĆ I. & J. FRANJIĆ 1994: Ass. *Salicetum elaeagno-daphnoides* (BR.-BL. et VOLK, 1940) M. MOOR 1958 (*Salicion elaeagni*) in the Vegetation in Croatia. Nat. Croat. **3** (2): 253-256.

WARD J. V. 1994a: Ecology of Alpine Streams. Freshwater Biology **32** (1): 10-15.

WARD J. V. 1994b: Ecology of Prealpine Streams. Freshwater Biology **32** (2): 10-15.

Knjige, poglavja iz knjig, poročila, kongresne povzetke citiramo sledeče:

ALLAN J. D. 1995: Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

PULLIN A. S., I. F. G. MCLEAN & M. R. WEBB 1995: Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: PULLIN A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150-164.

TOMAN M. J. 1992: Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 17.

12. Format in oblika članka

Članek naj bo napisan v programu *Word for Windows* ali *WordPerfect*, v pisavi "Times New Roman CE 12" z dvojnim medvrstnim razmakom in levo poravnavo ter s 3 cm robovi na A4 formatu. Odstavki naj bodo med seboj ločeni s prazno vrstico. Naslov članka in poglavij naj bodo pisani krepko in v velikosti pisave 14. Vsa latinska imena morajo biti napisana ležeče. V besedilu navedemo uporabljene nomenklaturne vire. Tabele in slike so posebej priložene tekstu. Glavnemu uredniku je potrebno oddati original, dve kopiji in disketni zapis na disketi 3,5" (odda avtor po opravljenih strokovnih in jezikovnih popravkih).

13. Recenzije

Vsak znanstveni članek bosta recenzirala dva recenzenta (en domači in en tuji), kratko notico pa domači recenzent. Avtor lahko v spremnem dopisu predlaga tuje recenzente. Recenziran članek, ki bo sprejet v objavo, popravi avtor. Po objavi prejme 50 brezplačnih izvodov. V primeru zavrnitve se originalne materiale vrne avtorju skupaj z negativno odločitvijo glavnega urednika.

INSTRUCTIONS FOR AUTHORS

1. Types of Articles

a) SCIENTIFIC ARTICLES are comprehensive descriptions of original research and include a theoretical survey of the topic, a detailed presentation of results with discussion and conclusion, and a bibliography according to the IMRAD outline (Introduction, Methods, Results, and Discussion). The length of an article including tables, graphs, and illustrations may not exceed fifteen (15) pages; lines must be double-spaced. Scientific articles shall be subject to peer review by two experts in the field.

b) REVIEW ARTICLES will be published in the journal after consultation between the editorial board and the author. Review articles may be longer than fifteen (15) pages.

c) BRIEF NOTES are original articles from various biological fields (systematics, biochemistry, genetics, microbiology, ecology, etc.) that do not include a detailed theoretical discussion. Their aim is to acquaint readers with preliminary or partial results of research. They should not be longer than five (5) pages. Brief note articles shall be subject to peer review by one expert in the field.

d) CONGRESS NEWS acquaints readers with the content and conclusions of important congresses and seminars at home and abroad.

e) ASSOCIATION NEWS reports on the work of Slovene biology associations.

2. Originality of Articles

Manuscripts submitted for publication in *Acta Biologica Slovenica* should not contain previously published material and should not be under consideration for publication elsewhere.

3. Language

Articles and notes should be submitted in English, or as an exception in Slovene if the topic is very local. As a rule, congress and association news will appear in Slovene.

4. Titles of Articles

Titles (in Slovene and English) must be short, informative, and understandable. The title should be followed by the name and full address of the author (and if possible, fax number and e-mail address).

5. Abstract

The abstract must give concise information about the objective, the methods used, the results obtained, and the conclusions. The suitable length for scientific articles is approximately 250 words, and for brief note articles, 100 words.

6. Keywords

There should be no more than ten (10) keywords; they must reflect the field of research covered in the article. Authors must add keywords in English to articles written in Slovene.

7. Introduction

The introduction must refer only to topics presented in the article or brief note.

8. Illustrations and Tables

Articles should not contain more than ten (10) illustrations (graphs, dendrograms, pictures, photos etc.) and tables, and their positions in the article should be clearly indicated. All illustrative material should be provided as physical originals (photographs or illustrations). Tables with their legends should be submitted on separate pages (only horizontal lines should be used in tables). Titles of tables should appear above the tables, and titles of photographs and illustrations below. Titles of tables and illustrations and their legends should be in both Slovene and English. Tables and illustrations should be cited shortly in the text (Tab. 1 or Tabs. 1-2, Fig. 1 or Figs. 1-2; Tab. 1 and Sl. 1).

9. Conclusions

Articles shall end with a summary of the main findings which may be written in point form.

10. Summary

Articles written in Slovene must contain a more extensive English summary. The reverse also applies.

11. Literature

References shall be cited in the text. If a reference work by one author is cited, we write ALLAN (1995) or (ALLAN 1995); if a work by two authors is cited, (TRINAJSTIĆ & FRANJIĆ 1994); if a work by three or more authors is cited, (PULLIN & al. 1995); and if the reference appears in several works, (HONSIG-ERLENBURG & al. 1992, WARD 1994a, ALLAN 1995, PULLIN & al. 1995). If several works by the same author published in the same year are cited, the individual works are indicated with the added letters a, b, c, etc.: (WARD 1994a,b). If direct quotations are used, the page numbers should be included: TOMAN (1992: 5) or (TOMAN 1992: 5-6).

The bibliography shall be arranged in alphabetical order beginning with the surname of the first author followed by the year of publication, the title of the article, the international abbreviation for the journal (periodical), the volume (in bold print), the number in parenthesis, and the pages. Examples:

HONSIG-ERLENBURG W., K. KRAINER, P. MILDNER & C. WIESER 1992: Zur Flora und Fauna des Weber-sees. Carinthia II **182/102** (1): 159-173.

TRINAJSTIĆ I. & J. FRANJIĆ 1994: Ass. *Salicetum elaeagno-daphnoides* (BR.-BL. et VOLK, 1940) M. MOOR 1958 (*Salicion elaeagni*) in the Vegetation in Croatia. Nat. Croat. **3** (2): 253-256.

WARD J. V. 1994a: Ecology of Alpine Streams. Freshwater Biology **32** (1): 10-15.

WARD J. V. 1994b: Ecology of Prealpine Streams. Freshwater Biology **32** (2): 10-15.

Books, chapters from books, reports, and congress anthologies use the following forms:

ALLAN J. D. 1995: Stream Ecology. Structure and Function of Running Waters, 1st ed. Chapman & Hall, London, 388 pp.

PULLIN A. S., I. F. G. MCLEAN & M. R. WEBB 1995: Ecology and Conservation of *Lycaena dispar*: British and European Perspectives. In: PULLIN A. S. (ed.): Ecology and Conservation of Butterflies, 1st ed. Chapman & Hall, London, pp. 150-164.

TOMAN M. J. 1992: Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 17.

12. Format and Form of Articles

Articles should be written with *Word for Windows* or *WordPerfect* using "Times New Roman CE 12" font with double spacing, align left and margins of 3 cm on A4 pages. Paragraphs should be separated with an empty line. The title and chapters should be written bold in font size 14. All scientific names must be properly italicized. Used nomenclature source should be cited. Tables and illustrations shall accompany the texts separately. The original manuscript, two copies, and a copy on a 3.5" computer diskette must be given to the editor-in-chief. All articles must be proofread for professional and language errors before submission.

13. Peer Review

All Scientific Articles shall be subject to peer review by two experts in the field (one Slovene and one foreign) and Brief Note articles by one Slovene expert in the field. Authors may nominate a foreign reviewer in an accompanying letter. Reviewed articles accepted for publication shall be corrected by the author. Authors shall receive fifty (50) free copies of the journal upon publication. In the event an article is rejected, the original material shall be returned to the author together with the negative determination of the editor-in-chief.

ČLANKI – ARTICLES: