ACTA BIOLOGICA SLOVENICA

VOL. 49 ŠT. 2 LJUBLJANA 2006

prej/formerly BIOLOŠKI VESTNIK

ISSN 1408-3671
UDK 57(497.4)

izdajatelj/publisher
Društvo biologov Slovenije

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2006 Vol. 49, Št. 2: 1–36

Acta Biologica Slovenica
Glasilo Društva biologov Slovenije – Journal of Biological Society of Slovenia

Izdaja – Published by
Društvo biologov Slovenije – Biological Society of Slovenia

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Acta Biologica Slovenica, Večna pot 111, SI-1001 Ljubljana, Slovenija
http://bijh.zrc-sazu.si/abs/

Oblikovanje – Design
Žare Vrezec

ISSN 1408-3671 UDK 57(497.4)

Natisnjeno – Printed on: 2007
Tisk – Print: Tiskarna Pleško d.o.o., Ljubljana
Naklada: 500 izvodov

Cena letnika (dve številki): 15 € za posameznike, 42 € za ustanove

Številka poslovnega računa pri Ljubljanski banki: 02083-142508/30

Publikacijo je sofinancirala Agencija za Raziskovalno dejavnost Republike Slovenije.

Acta Biologica Slovenica je indeksirana v – is indexed in: Biological Abstracts, Zoological records.

ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2006 Vol. 49, Št. 2: 3–12

Sprejeto (accepted): 2006-12-20

The RepFIIA replicon of the natural *Escherichia coli* plasmid pRK100

Replikon RepFIIA naravnega plazmida pRK100 bakterije *Escherichia coli*

Marjanca STARČIČ ERJAVEC & Darja ŽGUR-BERTOK

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111,
1000 Ljubljana, Slovenia, Fax: 00386 1 257 3390, E-mail: Marjanca.Starcic.Erjavec@bf.uni-lj.si,
Darja.Zgur@bf.uni-lj.si.

Abstract. The aim of the presented study was to identify the similarity of the plasmid pRK100 RepFIIA replicon (replication region) with similar replicons of other known plasmids of *Enterobacteriaceae*. For this purpose, within the determined nucleotide sequence of pRK100, the RepFIIA replicon *rep* genes/regions were identified. The nucleotide sequences of the pRK100 determined *rep* genes/regions were subsequently compared with the nucleotide sequences of other RepFIIA replicon *rep* genes/regions deposited in GenBank. Further, the nucleotide divergence between them was calculated. The obtained results clearly demonstrated, that the individual pRK100 *rep* regions are the same/most similar to *rep* regions from different plasmids. *RepA2* of pRK100 is most similar to *repA2* of pCP301, pINV_F6_M1382, pWR501 and R1, *copA* is the same as *copA* of plasmids pC15-1a and R100, *repA6* of pRK100 is the same as *repA6* in plasmids pC15-1a, pCP301, pINV_F6_M1382, pWR501, R1 and R100, *repA1* is most similar to *repA1* of the plasmid p1658/79, and *repA4* of pRK100 is most similar to *repA4* of pC15-1a. Hence, the composition of the pRK100 RepFIIA replicon is mosaic and unique among the plasmids.
Key words: plasmid, RepFIIA replicon, *Enterobacteriaceae*, nucleotide divergence

Izvleček. Cilj raziskave je bil označiti podobnost replikona (replikacijske regije) RepFIIA z drugimi podobnimi replikoni znanih plazmidov enterobakterij. V ta namen smo v nukleotidnem zaporedju replikona RepFIIA plazmida pRK100 poiskali posamezne gene/regije *rep* in njihovo nukleotidno zaporedje primerjali z drugimi, deponiranimi, nukleotidnimi zaporedji RepFIIA ter izračunali nukleotidno divergenco. Dobljeni rezultati so jasno pokazali, da so različni geni/regije *rep* v replikonu RepFIIA plazmida pRK100 enaki/zelo podobni *rep* različnih plazmidov. *RepA2* od pRK100 je najbolj podoben genu *repA2* plazmidov pCP301, pINV_F6_M1382, pWR501 in R1, *copA* je enak genu *copA* na plazmidih pC15-1a in R100, regija *repA6* plazmida pRK100 je enaka regiji *repA6* plazmidov pC15-1a, pCP301, pINV_F6_M1382, pWR501, R1 in R100, gen *repA1* je najbolj podoben genu *repA1* plazmida p1658/79, in regija *repA4* plazmida pRK100 je najbolj podobna regiji *repA4* plazmida pC15-1a. Povzamemo lahko, da je replikon RepFIIA plazmida pRK100 sestavljen kot mozaik in da ga v takšni sestavi do sedaj še niso našli na nobenem drugem plazmidu.
Ključne besede: plazmid, replikon RepFIIA, *Enterobacteriaceae*, nukleotidna divergenca

Introduction

Plasmids, extrachromosomal DNA elements, can be found in all three domains of the living world, in *Archaea*, *Bacteria* and *Eukarya* (Holmes & al. 1995, Solar & al. 1998, Zillig & al. 1998). These elements encode a remarkable array of phenotypic traits of medical, agricultural, environmental and commercial importance (Helinski & al. 1996). Encoded traits include resistances to heavy metals, supplementary metabolic pathways and pathways for degradation of xenobiotics, as well as virulence factors and resistances to antibiotics (Kado 1998). Further, plasmids can have the machinery to transfer themselves and other parts of the genome into different species, genera, or sometimes even families (Firth & al. 1996). Plasmids can also incorporate and deliver genes by recombination or transposition and by this means increase the genetic exchange in- and between bacterial populations (Solar & al. 1998).

All plasmids harbour a replicon (replication region), which is needed for the stable propagation and maintenance in the host cell. Regardless of plasmid size, the replicon of a plasmid generally consists of a contiguous set of information that includes a definable origin, where DNA replication initiates (*ori*), a structural gene encoding the plasmid-specific protein required for the initiation of replication, and one or more adjoining controlling elements. All this information is often contained within a segment that is 3 kb or less in size (Helinski & al. 1996). The replicons are designated and grouped into families (Couturier & al. 1988).

Replicons belonging to the RepFIIA family typically consist (Fig. 1) of *repA2* encoding a repressor, the *copA* gene that encodes an antisense RNA molecule, a *repA1* gene whose protein initiates plasmid replication by binding to the downstream *ori*, the *repA6* region encoding a short leader peptide, and a *repA4* region. The RepA2 repressor is assumed to regulate transcription of *repA1* mRNA, while the antisense RNA CopA which is complementary to the leader region of *repA1* mRNA (CopT), regulates translation. When CopA binds to CopT, *repA6,* which is necessary for RepA1 synthesis, is not expressed (Blomberg & al. 1992). The *repA4* appears to be important for the stability of plasmid maintenance (Jiang & al. 1993). Further it is known, that the replicons of this family are mosaic (Osborn & al. 2000), i.e. individual genes encoded in this replicon originate from different sources.

pRK100 is an ~145-kb plasmid isolated from a uropathogenic *Escherichia coli* strain and it has been to a large extent characterised. It belongs to the IncF incompatibility group and encodes two antibiotic resistances, ampicillin and tetracycline, two colicins, ColV and ColIa, and the aerobactin (*iuc*) and enterochelin (*iro*) iron uptake system. Further it was demonstrated that pRK100 harbours two different replicons, a RepFIB and RepFIIA replicon (Žgur-Bertok & al. 1990, Ambrožič & al. 1998, Starčič Erjavec, 2003). In the presented study the genes/regions of the pRK100 RepFIIA rep-

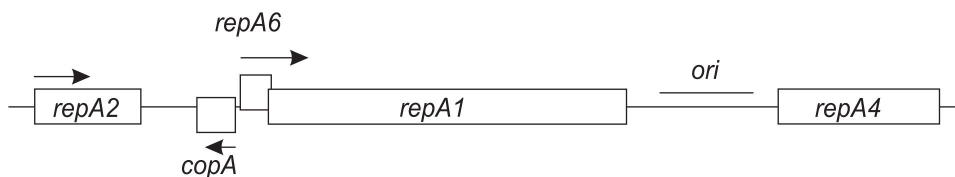

Fig. 1: Map of the RepFIIA replicon.
Genes/regions of a typical RepFIIA replicon are depicted. To clearly show the individual studied sequences, some boxes representing genes/regions are offline. The direction of mRNA transcription is also marked. *ori* is the origin of replication, where the RepA1 protein binds and starts the replication.

Slika 1: Mapa replikona RepFIIA.
Označeni so geni/regije tipičnega replikona RepFIIA. Zaradi razvidnosti lege preučevanih zaporedij, so nekateri okvirčki, ki prikazujejo gene/regije, premaknjeni. Označena je tudi smer prepisa mRNA iz posameznega gena. *ori* je regija, kjer se veže replikatorski protein RepA1 in prične s podvajanjem plazmida.

licon were analysed for their similarity with the RepFIIA replicon's genes/regions of related plasmids p1658/79, pB171, pC15-1a, pCP301, pINV_F6_M1382, pO157, pTUC100, pWR501, R1 and R100. The nucleotide divergence between pRK100 and the other plasmids was determined. The results of our study show that the RepFIIA replicon of pRK100 is mosaic and unique in its composition.

Method

Sequence analysis for open reading frames (ORF)

The determined RepFIIA replicon nucleotide sequence, 2159 bp in length, (GenBank accession number AY234375) was analyzed for open reading frames with the help of the program "ORF Finder" available on the web site http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi.

Sequence analysis for DNA similarity

The program Nucleotide-nucleotide BLAST (Altschul & al. 1997) available on the web site http://www.ncbi.nlm.nih.gov/BLAST/ was used to search for nucleotide sequences similar to the pRK100 RepFIIA nucleotide sequence. The DNA sequences of *rep* genes/regions were compiled and analysed using the CLUSTAL W (Thompson & al. 1994) program for sequence alignment. The program DNADIST in the PHYLIP package (Felsenstein 1993, Felsenstein 1989) was used for calculating distance matrixes.

Results

Genes/regions of the RepFIIA nucleotide sequence of pRK100

The pRK100 RepFIIA replicon has been deposited in GenBank under the Accession Number AY234377. In this deposited nucleotide sequence, the RepFIIA replicon is harboured from Nt 1401 to Nt 3559. To identify the *rep* genes/regions in the deposited nucleotide sequence of the pRK100 RepFIIA replicon, the internet program "ORF Finder" was used. To further define the *rep* genes/regions, the pRK100 RepFIIA sequence was compared to other known *rep* genes/regions of similar replicons. The RepFIIA replicon was found to harbour *repA2*, *copA*, *repA6*, *repA1*, and *repA4* sequences (Tab. 1).

Table 1: Predicted genes/regions in the pRK100 RepFIIA replicon (AY234377).
Tabela 1: Predvideni geni/regije replikona RepFIIA (AY234377) plazmida pRK100.

Assumed gene/region	Frame	From (bp)	To (bp)	Length
repA2	+3	1401	1661	261
copA	+1 C	1874	1782	93
repA6	+2	1886	1960	75
repA1	+3	1953	2810	858
repA4	+2	3173	3556	384

A pRK100 RepFIIA-like replicon can be found on many other plasmids

With the goal to find plasmids with similar replicons, which could be compared with the pRK100 RepFIIA replicon, a BLAST search with the nucleotide sequence of pRK100's RepFIIA was performed. The search revealed that many other plasmids carry similar replicons (Tab. 2). The most similar RepFIIA replicon was harboured by the *Escherichia coli* plasmid p1658/9. Most of the plasmids with similar replicon sequences were harboured on plasmids hosted by *Escherichia coli* or

Shigella flexneri however, some plasmids with less similar sequences were harboured also by other enteric bacterial species, as *Klebsiella pneumoniae*, *Shigella sonnei* and *Salmonella* Typhimurium. All plasmids exhibiting similarity with the pRK100 RepFIIA replicon belong to the broad RepFIIA family of replicons, those with higher similarity are members of the same Inc group – the IncFII, and plasmids with lower similarity belong to other Inc groups (IncFIA, IncFIC, IncFIV,..)

Table 2: BLAST hits for the nucleotide sequence of the pRK100 RepFIIA replicon with a score higher than 200.
Tabela 2: Zadetki z BLAST z rezultatom več kot 200 bitov za replikon RepFIIA plazmida pRK100.

GenBank Acc. Number	Plasmid	Host	BLAST score (bits)
AF550679	p1658/9	*Escherichia coli*	3733
V00351	R1(RSC13)	*Escherichia coli*	3527
AF177050	pWR100	*Shigella flexneri*	3213
AL391753	pWR100	*Shigella flexneri*	3166
AF348706	pWR501	*Shigella flexneri*	3166
AF386526	pCP301	*Shigella flexneri* 2a strain 301	3152
AY458016	pC15-1a	*Escherichia coli*	3019
AP000342	R100	*Shigella flexneri* 2b strain 222, *Escherichia coli*	2948
AY206448	pINV_F6_M1382	*Shigella flexneri*	2843
AB011549	pO157	*Escherichia coli*	2773
AB024946	pB171	*Escherichia coli*	2759
AY091607	pTUC100	*Escherichia coli*	2746
M26937	pSU316	*Escherichia coli*	2627
V00318	R6-5	*Escherichia coli*	926
M13472	ColV2-K94	*Escherichia coli*	924
X55895	pSU212	*Escherichia coli*	759
M93064	pEI545	*Klebsiella pneumoniae*	702
AP001918	F	*Escherichia coli*	420
M16167	P307	*Escherichia coli*	414
M28098	pSU221	*Escherichia coli*	394
M27528	R124	*Escherichia coli*	339
AP005147	R64	*Salmonella typhimurium*	311
AB021078	ColIb-P9	*Shigella sonnei* (*E. coli*)	303
K02675	pCG86	*Escherichia coli*	274
AP002527	R721	*Escherichia coli*	230

The pRK100 RepFIIA replicon is mosaic and unique in its composition

In order to identify the similarity of the pRK100 RepFIIA *rep* genes/regions with other known *rep* genes/regions the computer programs CLUSTAL W and DNADIST were used. With these programs nucleotide sequences of *rep* genes/regions of pRK100 with *rep* sequences of other similar RepFIIA replicons, which were found with BLAST search and gave a BLAST score of more then 1000 bits, were compiled. For the compilation only those BLAST hits that had a complete RepFIIA replicon nucleotide sequence deposited were used, so that entire RepFIIA replicon could be compared. For the comparison of RepFIIA genes/regions harboured by different plasmids the original genes/regions, if denoted by the submitters of the sequence, were used, otherwise the genes/regions in the deposited sequence were searched for using BLAST (Tab. 3). All together the nucleotide sequences of RepFIIA replicons of 10 plasmids (p1658/79, pB171, pC15-1a, pCP301, pINV_F6_M1382, pO157, pTUC100, pWR501, R1 and R100) were compared with the nucleotide sequence of pRK100 RepFIIA replicon.

The first region analysed in the RepFIIA replicon is the *repA2* gene, encoding a repressor protein, which by binding to the promoter represses the synthesis of *repA1* mRNA (Vanooteghem & Cornelis 1990). The *repA2* gene is in plasmids pRK100, p1658/79, pCP301, pINV_F6_M1382, pWR501, and R1 261 bp long, in plasmids R100, pO157, pC15-1a and pTUC100 it is 255 bp long and in the plasmid

Table 3: Plasmids and their *rep* sequences used in CLUSTAL W and DNADIST computer programs.
The studied sequences are denoted either according to the data in GenBank or marked as BLAST to show that the sequence was searched for by BLAST. For each studied sequence the position on the deposited sequence and the length are given.

Tabela 3: Plazmidi in njihova zaporedja *rep*, ki smo jih uporabili v računalniških programih CLUSTAL W in DNADIST.
Preučevana zaporedja so označena tako kot so podana v podatkovni bazi GenBank oziroma označena z BLAST, v primeru če so bila poiskana z BLAST. Za vsako preučevano zaporedje je navedena njegova pozicija na deponiranem zaporedju in njegova dolžina.

	repA2	***length (bp)***	***copA***	***length (bp)***	***repA6***	***length (bp)***	***repA1***	***length (bp)***	***repA4***	***length (bp)***
p1658/79	BLAST: 37469–37729	261	BLAST: *c* 37850–37943	94	repA6: 37955–38029	75	repA1: 38011–38879	869	repA4: 39243–39449	207
pB171	copB: *c* 39420–39668	249	BLAST: *c* 39185–39278	94	BLAST: 39099– 39173	75	repA1: *c* 38249–39106	858	BLAST: *c* 37500–37887	388
pC15–1a	repA2: 88558–88812	255	BLAST: *c*88946–89038	93	repA6: 89050–89124	75	repA1: 89117–89974	858	repA4: 90337–90723	387
pCP301	repB: 208897–209157	261	BLAST: *c* 209278–209369	92	tapA: 209381–209455	75	repA: 209436–210305	870	BLAST: 210668–211049	383
pINV_F6–_M1382	repB: 831–1091	261	BLAST: *c* 1212–1303	92	tap: 1315–1389	75	repA: 1382–2239	858	BLAST: 2602–2988	387
pO157	repA2: 71999–72253	255	BLAST: *c* 72387–72477	91	BLAST: 72489–72563	75	repA1: 72556–73416	858	BLAST: 73775–74158	384
pTUC100	repA2: *c*5976–6230	255	repA3: 5744–5872	129	repA6: *c* 5665–5739	75	repA1: *c* 4815–5672	858	repA4: *c* 4066–4452	387
pWR501	repA2: 208603–208863	261	S0294 (inc–RNA): *c* 208980–209069	90	repA6: 209087–209161	75	repA1: 209142–210011	870	BLAST: 210374–210756	382
R1	repA2: 442–702	261	BLAST: *c* 823–915	93	BLAST: 927–1001	75	repA1: 994–1851	858	repA4: 2214–2600	387
R100	repA2: 88253–88507	255	inc: *c* 88641–88733	93	repA6: 88745–88819	75	repA1: 88812–89669	858	repA4: 90032–90418	387

pB171 it is only 249 bp long. The sequence analysis showed, that pRK100 *repA2* is most similar to *repA2* of pCP301, pINV_F6_M1382, pWR501 and R1, since the phylogenetic distance between them is the smallest, namely 0,0038 (Fig. 2A).

The second region analysed in the RepFIIA replicon is the *copA* gene, encoding the antisense RNA regulating the translation of *repA1* mRNA (Vanooteghem & Cornelis 1990). The *copA* gene is in most of the compared plasmids approximately 90 bp long. The only exception is the *copA* gene of the plasmid pTUC100, which is 129 bp long. The sequence analysis showed, that there is no nucleotide divergence between pRK100 *copA* and the copA genes of R100 and pC15-1a (Fig. 2B), and hence the pRK100 *repA* gene is identical to the appropriate genes in R100 and pC15-1a.

The third region analysed in the RepFIIA replicon is the *repA6*. *repA6* encodes a short leader peptide, whose expression is inhibited by CopA binding, preventing translation of RepA and consequently preventing plasmid replication. (Bloomberg & al. 1992). The *repA6* is only 75 nucleotides long and it has the same size in all compared plasmids. The compared nucleotide sequences of pRK100 *repA6* was completely identical (no nucleotide divergence) to *repA6* of plasmids pC15-1a, pCP301, pINV_F6_M1382, pWR501, R1 and R100 (Fig. 2C).

The fourth region analysed in the RepFIIA replicon is *repA1* gene, encoding the RepA protein needed for the plasmid's replication (Helinski & al. 1996). The *repA1* genes of plasmids pB171, pC15-1a, pINV_F6_M1382, pO157, pTUC100, R1 and R100 are 858 bp long, the *repA1* of p1658/79 is 869 bp long, and the *repA1* genes of plasmids pCP301 and pWR501 are 870 bp long. The phylogenetic distance between the pRK100 *repA1* nucleotide sequence and the *repA1* of plasmid p1658/79 is the smallest, only 0,0154 (Fig. 2D), hence the pRK100 *repA1* is most similar to *repA1* of plasmid p1658/79.

The fifth and the last region analysed in the RepFIIA replicon, is *repA4*. Though it encodes no product, it is important for the stability of plasmid maintenance (Jiang & al. 1993). The p1658/79 *repA4* is 207 bp long, while the *repA4* sequences of other compared plasmids are around 385 bp long. The phylogenetic distance between the pRK100 *repA4* nucleotide sequence and *repA4* of plasmid pCP15-1a is the smallest, 0,0520 (Fig. 2E), therefore the *repA4* nucleotide sequence of pRK100 is most similar to *repA4* of plasmid pCP15-1a.

Since the individual pRK100 *rep* genes/regions are similar to *rep* genes/regions of different plasmids, it can be concluded, that the pRK100 RepFIIA replicon is mosaic in structure and unique among the RepFIIA replicons.

Discussion

Plasmid replicons are essential for plasmid maintenance in the host cell. The replicons can differ with regard to their replication control mechanisms, origin of replication sequences and replication proteins. Plasmids with very similar origin sequences and replication control mechanisms are assigned into families. The pRK100 replicon described in this article belongs to the RepFIIA family of replicons.

In order to characterise the RepFIIA replicon of pRK100 its nucleotide sequence was first searched for genes/regions. Each RepFIIA replicon consists of five genes/regions, *repA2* gene, *copA* gene, *repA6* region, *repA1* gene and *repA4* region. With the help of the computer programs "ORF Finder" and BLAST the five genes/regions in the pRK100 RepFIIA replicon were determined, however the genes are only putative and more experimental work is needed to confirm the predicted gene lengths and their functions.

To elucidate the similarity of the pRK100 RepFIIA replicon with other known RepFIIA replicons, a BLAST search on the complete pRK100 RepFIIA nucleotide sequence was performed. More then 170 BLAST hits were found, most of them belonging to replicon sequences of plasmids from *Enterobacteriaceae.* This is not surprising as the broad RepFIIA replicon family is known to be highly prevalent in enteric bacteria (Osborn & al. 2000).

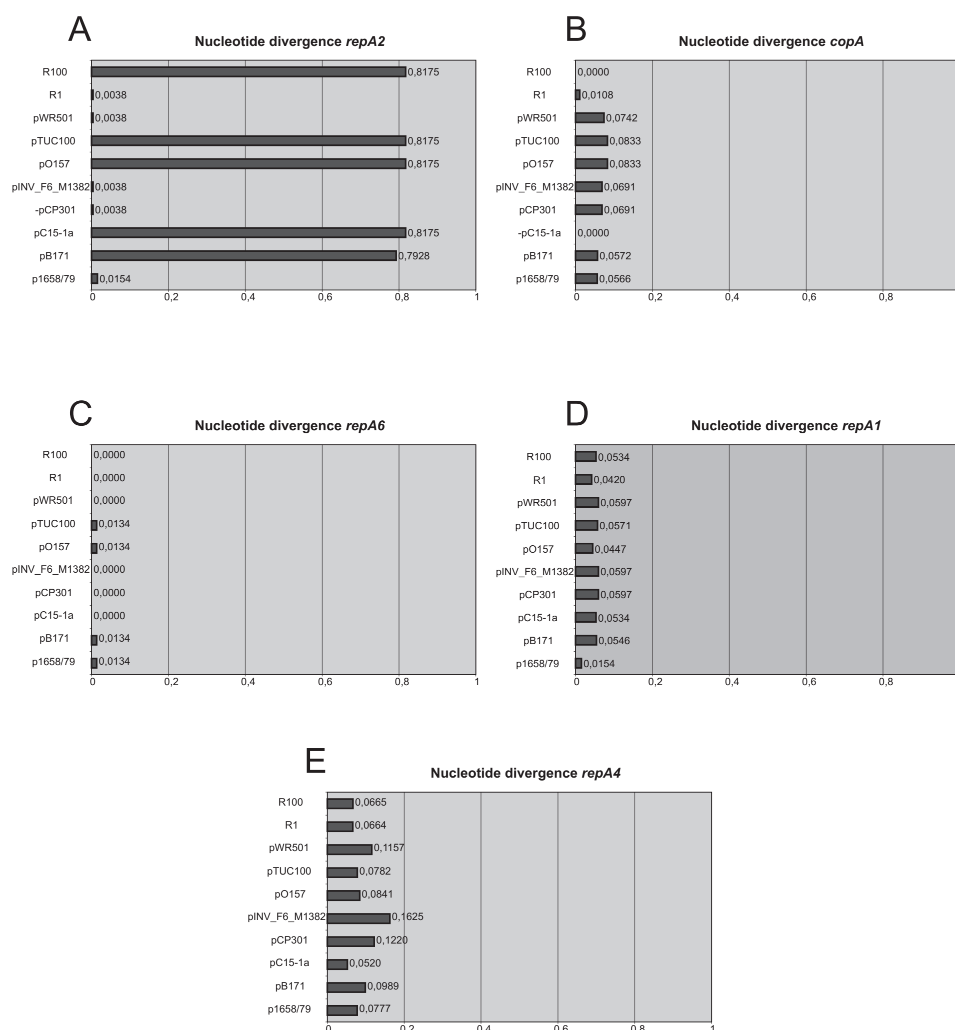

Fig. 2: Nucleotide divergence of pRK100 RepFIIA *rep* gene/region sequences and related sequences.
Nucleotide divergence, as the measurement of the phylogenetic nucleotide distance, was calculated according to the Kimura-2 parameter. Nucleotide divergences between pRK100 sequences and sequences of other related plasmids are plotted: Panel A – nucleotide divergence of *repA2*, panel B – nucleotide divergence of *copA*, panel C – nucleotide divergence of *repA6*, panel D – nucleotide divergence of *repA1*, and panel E – nucleotide divergence of *repA4.*

Slika 2: Nukleotidna divergenca dobljenih zaporedij genov/regij *rep* replikona RepFIIA plazmida pRK100 in sorodnih zaporedij.
Nukleotidna divergenca, kot mera za filogenetsko razdaljo, je bila izračunana po parametru Kimura-2. Nukleotidne divergence med zaporedji plazmida pRK100 in ostalimi sorodnimi zaporedji so prikazana: graf A – nukleotidna divergenca *repA2*, graf B – nukleotidna divergenca *copA*, graf C – nukleotidna divergenca *repA6*, graf D – nukleotidna divergenca *repA1* in graf E – nukleotidna divergenca *repA4.*

The most relevant BLAST hits (BLAST score more then 1000 bits) were used for a detailed similarity analysis. The chosen RepFIIA replicons to be compared with the pRK100 replicon were harboured by plasmids p1658/79, pB171, pC15-1a, pCP301, pINV_F6_M1382, pO157, pTUC100, pWR501, R1 and R100. Since it is known, that the RepFIIA replication family is mosaic in its composition (Osborn & al. 2000), each *rep* region was analysed separately. Also from our analysis the mosaic structure of the pRK100 RepFIIA replicon is evident, since *RepA2* of pRK100 is most similar to *repA2* of pCP301, pINV_F6_M1382, pWR501 and R1, *copA* is the same as *copA* of plasmids pC15-1a and R100, the *repA6* of pRK100 is the same as *repA6* in plasmids pC15-1a, pCP301, pINV_F6_M1382, pWR501, R1 and R100, *repA1* is the most similar to *repA1* of plasmid p1658/79, and *repA4* of pRK100 is the most similar to *repA4* of pC15-1a. Further, it can also be concluded, that the composition of the pRK100 RepFIIA replicon is unique.

Even though all nucleotide sequences of genes/regions incorporated into this study, belong to the same replicon, the RepFIIA, the nucleotide divergence between different genes/regions is not the same, thus the *repA6* region is very conserved, while other genes, as *copA* and *repA1*, are less conserved. A higher level of nucleotide divergence was observed in the *repA4* sequence. This fact is not surprising, since this sequence has no product and hence no product-connected selection can influence the evolution of this sequence. However, the greatest differences in nucleotide divergence were observed in the *repA2* gene, encoding the repressor. It might be assumed that the observed differences are important for plasmid incompatibility and it would be very interesting to test the ability of the studied plasmids to propagate in the same host.

The observed mosaicism of the pRK100 RepFIIA replicon is not the only example of a mosaic sequence of pRK100. In a previous report (Starčič Erjavec & al. 2002) we also demonstrated, that the pRK100 *tra* region is mosaic. However, overall the studied *tra* region genes were most similar to the *tra* genes of plasmid F, but in the case of the pRK100 RepFIIA replicon, no overall similarity with only one plasmid could be detected. This again illustrates, that plasmid genes are mosaic and formed by multiple recombination events between diverse ancestral genes (Boyd & al. 1996).

Conclusions

To summarise and conclude:

1. the pRK100 RepFIIA replicon harbours 5 genes/regions *repA2*, *copA*, *repA6*, *repA1* and *repA4;*
2. the individual *rep* genes/regions of the pRK100 RepFIIA replicon exhibit different nucleotide divergence when compared with different related plasmids;
3. the pRK100 RepFIIA replicon is mosaic and unique;
4. many other plasmids of *Enterobacteriaceae* carry replicons similar to pRK100 RepFIIA replicon.

Povzetek

V predstavljeni raziskavi smo na nivoju nukleotidnih zaporedij preučevali podobnost replikona RepFIIA plazmida pRK100 z replikoni RepFIIA ostalih sorodnih plazmidov. S pomočjo računalniških programov “ORF Finder” in BLAST smo na zaporedju replikona RepFIIA plazmida pRK100 poiskali replikacijske gene/zaporedja *repA2*, *copA*, *repA6*, *repA1* in *repA4.* Z računalniškima programoma CLUSTAL W in PHYLIP smo predvidene replikacijske gene/zaporedja primerjali z replikacijskimi geni/zaporedji drugih sorodnih plazmidov (p1658/79, pB171, pC15-1a, pCP301, pINV_F6_M1382, pO157, pTUC100, pWR501, R1 in R100), ki so deponirani v GenBank in smo jih poiskali z računalniškim programom BLAST. Na podlagi dobljenih nukleotidnih divergenc je razvidno, da je gen

RepA2 od pRK100 najbolj podoben genu *repA2* plazmidov pCP301, pINV_F6_M1382, pWR501 in R1, *copA* je enak genu *copA* na plazmidih pC15-1a in R100, regija *repA6* plazmida pRK100 je enaka regiji *repA6* plazmidov pC15-1a, pCP301, pINV_F6_M1382, pWR501, R1 in R100, gen *repA1* je najbolj podoben genu *repA1* plazmida p1658/79, in regija *repA4* plazmida pRK100 je najbolj podobna regiji *repA4* plazmida pC15-1a. Če povzamemo vse rezultate, lahko zaključimo, da je replikon RepFIIA plazmida pRK100 sestavljen kot mozaik in da ga v takšni sestavi do sedaj še niso našli na nobenem drugem plazmidu.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2006 Vol. 49, Št. 2: 13–21

Sprejeto (accepted): 2006-09-28

Colicins of the *Escherichia coli* uropathogenic strain collection

Kolicini zbirke uropatogenih bakterij *Escherichia coli*

Marjanca STARČIČ ERJAVEC, Matija RIJAVEC, Darja ŽGUR-BERTOK

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia, Fax: 00386 1 257 3390, E-mail: Marjanca.Starcic.Erjavec@bf.uni-lj.si, r_matija@yahoo.com, Darja.Zgur@bf.uni-lj.si.

Abstract. 110 uropathogenic *Escherichia coli* (UPEC) strains were screened for colicin production and 42 (38%) of the tested UPEC strains were found to be colicinogenic. The ColA, ColB, ColD, ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColIa, ColIb, ColK, ColN, MccB17, ColS4, MccC7 and ColE6-J colicin producer strains from Pugsley's collection of colicinogenic strains were lysed by all colicinogenic UPEC strains, the ColM and ColE1 producer strains by 93% of the UPEC colicinogenic strains and the ColV producer strain by only 81% of the UPEC colicinogenic strains. 67% of the colicinogenic UPEC strains were able to lyse all 20 used colicin producer strains and 33% of the colicinogenic UPEC strains were able to lyse 19 Pugsley's strains. Hence, a majority (67%) of the studied UPEC strains encode and produce either more than one colicin, or a colicin not tested. Colicins of UPEC strains producing only one colicin were identified; 8 strains (19% of the colicinogenic strains) produced only ColV, 3 strains (7%) ColM and 3 strains (7%) ColE1. Plasmids were found in 88% of the colicinogenic strains. 11 DL strains were found to harbour conjugative plasmids encoding antibiotic resistance(s) and colicinogenicity. Further, 19% of the haemolytic UPEC strains and 44% of non-haemolytic strains were also colicinogenic, 28% of the *cnf* encoding strains and 41% of the strains not encoding *cnf* were colicinogenic, while 40% of *ibeA* encoding strains and 38% of strains not encoding *ibeA* were colicinogenic.
Key words: colicin, uropathogenic *Escherichia coli*, UPEC, plasmid, haemolysin, *hly*, cytotoxic necrotising factor, *cnf*, invasin, *ibeA*.

Izvleček. 110 uropatogenih sevov bakterije *Escherichia coli* (UPEC) smo s pomočjo 20 kolicinogenih sevov iz Pugsleyeve zbirke testirali za produkcijo kolicinov. 42 testiranih sevov (38%) je bilo kolicinogenih. Vsi kolicinogeni sevi UPEC so povzročili propad producentskih sevov kolicinov ColA, ColB, ColD, ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColIa, ColIb, ColK, ColN, MccB17, ColS4, MccC7 in ColE6-J iz Pugsleyeve zbirke. 93% kolicinogenih sevov UPEC je povzročilo propad ColM in ColE1 producentskega seva in 81% kolicinogenih sevov UPEC je povzročilo propad producentskega seva kolicina ColV. 67% kolicinogenih sevov UPEC je povzročilo propad vseh 20 kolicinogenih sevov Pugsleyeve zbirke in 33% kolicinogenih sevov UPEC je povzročilo propad 19 sevov iz Pugsleyeve zbirke. Večina kolicinogenih sevov (67%) je sintetizirala vsaj dva kolicina, ali pa kolicin, ki ni bil testiran. Kolicine sevov UPEC, ki sintetizirajo samo 1 kolicin, smo prepoznali; 8 sevov UPEC (19%) je sintetiziralo samo ColV, 3 sevi (7%) so sintetizirali samo ColM in 3 sevi (7%) so sintetizirali samo ColE1. V 88% kolicinogenih sevov smo našli plazmide. V 11 sevih DL smo našli konjugativne plazmide z zapisi za odpornost proti antibiotikom in za sintezo kolicinov. 19% hemolitičnih sevov UPEC in 44% nehemolitičnih sevov UPEC je bilo kolicinogenih; 28% sevov z zapisom *cnf* in 41% sevov brez

zapisa *cnf* je bilo tudi kolicinogenih, medtem ko je bilo 40% z zapisom *ibeA* in 38% brez tega zapisa tudi kolicinogenih.

Ključne besede: kolicin, uropatogena *Escherichia coli*, UPEC, plazmid, hemolizin, *hly*, citotoksičen nekrotizirajoč dejavnik, *cnf*, invazin, *ibeA*.

Introduction

Colicins are bacteriocins produced by *Escherichia coli* (*E. coli*) strains. As other bacteriocins, produced by different types of *Eubacteria* and *Archaebacteria* (Riley & Gordon 1999), colicins are extracellular bacterial toxic proteins, that are active against the same species, or closely related species of the producer cell (Daw & Falkiner 1996). The mechanism of action of these compounds involves adsorption to specific receptors located on the external surface of sensitive bacteria followed by killing via one of three primary mechanisms: the formation of channels in the cytoplasmic membrane, the degradation of cellular DNA or the inhibition of protein synthesis (Riley & Gordon 1999). Because of their narrow range of activity, it has been proposed that the primary role of bacteriocins is to mediate intraspecific, or population level, interactions (Riley 1998).

However, bacteriocins have been also implicated in virulence determination, since many pathogenic strains harbour plasmid-encoded bacteriocins, for example ColV (Waters & Crosa 1991). A relatively high frequency of colicin producing strains is found in isolates of pathogenic *E. coli* (van der Wal & al. 1995), for example approximately 80% of enterohemorrhagic *E. coli* strains studied by Bradley and Howard were colicinogenic (Bradley & Howard 1991).

In the presented study a collection of 110 uropathogenic *E. coli* (UPEC) strains, isolated at the Institute of Microbiology and Immunology of the Medical Faculty of Ljubljana, Slovenia, was examined for the ability to produce colicins and lyse other *E. coli* strains of the Pugsley collection of colicinogenic strains. Further, the association of colicinogenicity and some established virulence factors was also analysed. The results of the study show, that almost 40% of the UPEC strains are colicinogenic and that all colicinogenic strains are very efficient in lysing *E. coli* strains of the Pugsley collection. Further, a non-proportional distribution of the ability to produce colicins among the *hly* and *cnf* coding versus non-coding strains was determined. In 88% of the colicinogenic strains plasmids were found, 11 colicinogenic strains harboured conjugative plasmids encoding antibiotic resistances and colicin production.

Methods

Bacterial strains, plasmids and growth conditions

The 110 uropathogenic *Escherichia coli* DL strains used in this study were isolated from urine of patients with urinary tract infections at the Institute of Microbiology and Immunology of the Medical Faculty of Ljubljana. The strain AB1133 [*thr1 leuB6 proA2 his argE2 thi ara lacY galK2 xyl mtl rpsL* α^- *supE* (B. Bachmann)], which is sensitive to all colicins, was used to identify colicinogenic UPEC strains. The strains of Pugsley's collection of colicinogenic strains are listed in Tab. 1. For mating experiments the following strains were used: DH5α [Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* (BRL Life)], HB101 [*hsdR hsdM recA13 supE44 leuB6 lacZ proA2* (D. Ehrlich)], RU4404 [MM294::Tn*1725* Cm^r *thi endA hsdR* (R. Schmitt)], RU4406 [MM294::Tn*1732* Kn^r *thi endA hsdR* (R. Schmitt)] and TR51 [*ara*D139 Δ(*argF-lac*)U169 *rps*L150 *rel*A1 *flb*B5301 *pts*F25 *deo*C1 *cpxR*::*spc* Spc^r (T. J. Silhavy)]. Bacteria were grown in Luria-Bertani (LB) medium with aeration at 37°C or on LB plates without aeration. Ampicillin (Ap, 100 μg/ml), tetracycline (Tc, 10 μg/ml), kanamycin (Kn, 30 μg/ml), chloramphenicol (Cm, 50 μg/ml), spectinomycin (Spc, 20 μg/ml), streptomycin (Sm, 150 μg/ml), trimethoprim (Tp, 10 μg/ml) and nalidixic acid (Nal, 25 μg/ml) were added to the growth media, when appropriate.

Table 1: Bacterial strains of Pugsley's collection.
Tabela 1: Bakterijski sevi Pugsleyeve zbirke.

Strain	Relevant features
BZB2101	ColA producer
BZB2102	ColB producer
BZB2103	ColD producer
BZB2104	ColE1 producer
BZB2125	ColE2 producer
BZB2106	ColE3 producer
BZB2107	ColE4 producer
BZB2108	ColE5 producer
BZB2109	ColE6 producer
BZB2110	ColE7 producer
BZB2114	ColIa producer
BZB2115	ColIb producer
BZB2116	ColK producer
BZB2123	ColN producer
BZB2283	MccB17 producer
PAP1	ColM producer
PAP2	ColS4 producer
PAP54	MccC7 producer
PAP222	ColV producer
PAP247	ColE8-J producer

Overlay test

The overlay test was based on the method described by Pugsley and Oudega (1987). The UPEC strains were inoculated on LB plates (20 colonies grid) using toothpicks. Following overnight incubation the cells were lysed with chloroform, to release the colicins. After aeration, the plates were overlaid with 4 ml of soft agar with 0,2 ml of an overnight culture of either AB1133 or one of the strains from Pugsley's collection. The plates were then incubated overnight and next day examined for zones of colicin activity (clear zones around the colonies).

Plasmid isolation

Plasmid DNA was prepared by the alkaline lysis method described in Sambrook et al. (1989).

Mating assay

Conjugation experiments were performed overnight on LB plates. The mating mixture was transferred to LB plates supplemented with appropriate antibiotics to select for transconjugants. The conjugative plasmids encoding antibiotic resistances were initially, depending on the antibiotic resistance profile, transferred to either DH5α, HB101, RU4404, RU4406 or TR51 and further to a another laboratory strain to confirm the self conjugative transfer ability. All conjugative plasmids were finally transferred to strain DH5α.

Results

Colicinogenic UPEC strains and their colicins

In order to identify the colicinogenic UPEC strains, strain AB1133, which is known to be sensitive to all colicins, was used in an overlay test as described above. 42 UPEC strains (38%) were able to lyse strain AB1133, hence exhibited colicinogenic activity. To attempt to characterise colicin encoded by

the individual UPEC strains, the 20 strains of Pugsley's collection of colicin producing strains were used in overlay tests. The tests showed, that most of the colicinogenic UPEC strains (67%) produced either more than one colicin, or a colicin, not harboured by strains of the Pugsley collection. However, 14 UPEC strains were shown to produce only one colicin, namely 8 strains (19% of the colicinogenic strains) produced only ColV, 3 strains (7%) ColM and 3 strains (7%) produced only ColE1 (Tab. 2).

Table 2: Colicinogenic DL strains.
Tabela 2: Kolicinogeni sevi DL.

Strain	Number of lysed Pugsley's strains	Identified colicin	Plasmid detected	Conjugative plasmid
DL2	20		+	+
DL3	20		+	–
DL6	20		+	+
DL10	20		+	–
DL14	20		+	+
DL22	20		+	+
DL24	20		+	–
DL27	19	ColV	+	–
DL35	20		+	–
DL37	20		+	+
DL40	19	ColM	–	–
DL46	20		+	+
DL48	20		+	+
DL49	20		+	–
DL51	20		+	–
DL52	19	ColV	+	–
DL53	20		+	–
DL56	20		+	+
DL57	19	ColV	+	–
DL58	20		+	–
DL59	20		–	–
DL60	20		+	–
DL62	19	ColV	–	–
DL63	19	ColE1	+	–
DL64	19	ColV	+	–
DL66	19	ColV	+	–
DL67	19	ColV	+	–
DL71	19	ColE1	+	–
DL72	19	ColE1	+	–
DL75	20		+	–
DL76	20		+	+
DL83	19	ColV	+	–
DL89	19	ColM	+	–
DL91	19	ColM	+	–
DL92	20		–	–
DL93	20		+	–
DL95	20		–	–
DL99	20		+	–
DL104	20		+	–
DL107	20		+	–
DL108	20		+	+
DL110	20		+	+

Sensitivity of Pugsley's colicinogenic strains for the colicinogenic UPEC strains

The sensitivity of Pugsley's colicinogenic strains for the colicinogenic UPEC strains was also demonstrated in the overlay tests. The ColA, ColB, ColD, ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColIa, ColIb, ColK, ColN, MccB17, ColS4, MccC7 and ColE6-J colicin producer strain from Pugsley's collection were lysed by all colicinogenic UPEC strains, the ColM and ColE1 producer strains by 93% of the UPEC colicinogenic strains and the ColV producer strain by only 81% of the UPEC colicinogenic strains (Graph 1).The high sensitivity of the Pugsley colicinogenic strains for the colicinogenic UPEC strains is also evident from Tab. 2, which shows that 67% of the colicinogenic UPEC strains were able to lyse all 20 of the used colicin producer strains and 33% of the colicinogenic UPEC strains were able to lyse 19 of the Pugsley strains.

The ability to produce colicins and plasmids

Preparation of plasmid DNA with alkaline lysis from colicinogenic strains revealed that 37 (88%) of the colicinogenic strains harboured plasmids. In only 5 strains (DL40, DL59, DL62, DL92 and DL95) no plasmid DNA was detected (Tab. 2).

Colicinogenic proprieties encoded on conjugative plasmids

In the mating assays, 19 DL strains (DL2, DL6, DL7, DL8, DL14, DL17, DL22, DL37, DL41, DL43, DL46, DL48, DL56, DL76, DL81, DL84, DL108, DL109 and DL110) were found to harbour conjugative plasmids encoding antibiotic resistances. 11 of these strains (DL2, DL6, DL14, DL22, DL37, DL46, DL48, DL56, DL76, DL108 and DL110) were also found to be colicinogenic (Tab. 2). To determine whether the colicinogenicity is encoded on a conjugative plasmid (pDL), the DH5α laboratory strains harbouring the conjugative plasmids from the DL strains were subjected to overlay tests. When the DH5α strains harbouring pDL conjugative plasmids were overlaid with the Pugsley strains, all 20 of the Pugsley strains were lysed. Further, when the original DL strains were overlaid by DH5α strains harbouring pDL conjugative plasmids, the DH5α pDL strains were not lysed by the original DL strain. The obtained results clearly demonstrated that colicinogenicity in all of these strains was encoded by a conjugative plasmid and hence, was transferable to other strains.

The ability to produce colicins and some other virulence factors

The obtained data concerning colicinogenicity of the tested strains presented in this paper were compared with our unpublished data on the prevalence of virulence factors; namely haemolysin (*hly*), cytotoxic necrotising factor (*cnf*) and invasin (*ibeA*). Our results showed that 19% of the haemolytic UPEC strains and 44% of the non-haemolytic strains were also colicinogenic, 28% of the *cnf* encoding strains and 41% of the strains not encoding *cnf* were colicinogenic, while 40% of the *ibeA* encoding strains and 38% of the strains not encoding *ibeA* were colicinogenic (Fig. 1).

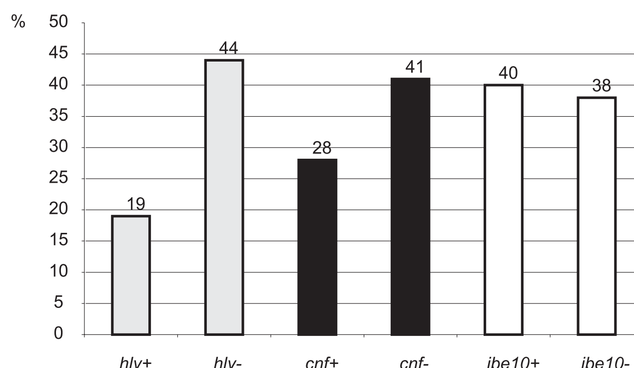

Fig. 1: Incidence of colicinogenic strains in correlation with some virulence factors. The incidence of colicinogenic strains is expressed in percentage of strains encoding or not encoding haemolysin (*hly*), cytotoxic necrotising factor (*cnf*) and invasin (*ibeA*).

Slika 1: Pogostnost kolicinogenih sevov v povezavi z nekaterimi virulenčnimi dejavniki. Pogostnost kolicinogenosti je izražena v odstotkih sevov z oziroma brez zapis za hemolizin (*hly*), citotoksičen nekrotizirajoč dejavnik (*cnf*) in invazin (*ibeA*).

Discussion

The DL strain collection of uropathogenic *E. coli* strains was screened for colicin production. 38% of the examined strains exhibited in the performed overlay tests a phenotype consistent with colicin production. Since the overlay tests were performed without mitomycin C treatment, which is known to induce temperate bacteriophage, it can be assumed that the obtained results have not been misinterpreted due to bacteriophage. However, to completely rule out the possibility of misinterpretation of bacteriophage activity as colicin production further tests would be needed.

Typically, 25–50% of *E. coli* isolates are colicinogenic (Riley & Gordon 1996). Usually, the percentages are higher in pathogenic than commensal strains, they are also higher in human compared to animal strains (Riley & Gordon 1996). In this study the obtained percentage (38%) of colicin producing strains in the DL collection is comparable to that of other studies, McGeachie (1965) found 36% of colicinogenic strains among uropathogenic *E. coli* strains and O'Brien et al. (1996) found 41% of colicin producers among uropathogenic *E. coli* strains. The obtained percentage is also comparable to the incidence of colicinogenicity (41%) in strains from colons of healthy persons (Šmarda & Obdržalek 2001).

It is established that the frequency of different colicin types varies substantially between populations (Riley & Gordon 1996), hence the colicin pattern in the DL collection should differ from patterns in other *E. coli* collections. The applied tests allowed the assignment of colicins of those strains producing only one colicin, namely ColV by 19% of the colicinogenic strains, ColM and ColE1 by 7% of the colicinogenic strains. Colicins of all other colicinogenic strains (67%) could not be assigned as these strains produce more than one colicin or they produce a colicin type not presented in the Pugsley collection of colicinogenic strains. However, the combination of ColV, ColM and ColE1 colicins in one collection is, to our knowledge, unique.

High levels of colicin resistance are known to occur in natural *E. coli* populations (Feldgarden & Riley 1998), hence the high sensitivity of Pugsley's collection for the colicinogenic strains (the majority, 67%, of colicinogenic strain were able to lyse all 20 strains of Pugsley's collection), is a bit surprising. But it is explainable with the assumption, that the UPEC strain encode more than one colicin or a colicin not produced by the Pugsley strains collection.

It is known, that the genetic determinants of most of the colicins are located on the plasmids, apart from few, which are chromosomally encoded (Daw & Falkiner 1996). Hence, it is not surprising that in 88% of colicinogenic strains we were able to detect plasmid DNA. A very similar percentage, 86%, of plasmid harbouring colicin producing strains was found by Riley and Gordon (1992) in the ECOR collection, a collection of strains representing clinical and non-clinical isolates from man, domestic and zoo animals.

To determine whether the colicins are really encoded on the detected plasmids, the plasmids should be transferred to laboratory strains and subsequently tested for colicin production. Unfortunately, the capabilities of transferring the plasmids to laboratory strains are limited due to large plasmid size and lack of selection possibilities. However, several conjugative plasmids encoding antibiotic resistances were found in the DL collection and were successfully transferred. The overlay tests performed on these strains showed that all such plasmids (11) also encoded colicin production.

The role of colicins in microbial communities is still not clear (Riley & Wertz 2002). Bacteriocins may serve as anti-competitors enabling the invasion of a strain into an established microbial community or they may play a defensive role and act to prohibit the invasion of other strains or species into an occupied niche or limit the advance of neighbouring cells (Riley & Wertz 2002). Further it was also suggested that colicins, at least ColV, more exactly the virulence factors also encoded by ColV plasmids might play a role in pathogenesis (Waters & Crosa 1991). In a study of UPEC strains Šmarda & Obdržalek (2001) observed that the incidence of colicinogenicity significantly differs between haemolytic and non-haemolytic strains (42% of non-haemolytic and only 22% of haemolytic strains were colicinogenic). A comparable difference was also observed in the presented study, as 44% of the non-haemolytic and 19% of the haemolytic strains were colicinogenic. Further, a similar even though less distinctive difference, was observed with the *cnf* encoding (28%) and *cnf* non encoding strains (41%). These results suggest, that the production of colicins is associated with some virulence factors, since otherwise the incidence of colicinogenic strains should be equal in both groups, as it is in the presented study for the *ibeA* encoding (40%) and non *ibeA* encoding strains (38%). Even though the obtained results could indicate that colicinogenic strains are less virulent, further studies are needed to establish the role colicins play in natural populations.

Conclusions

To summarise and conclude:

1. 110 uropathogenic *Escherichia coli* (UPEC) strains were screened for colicin production;
2. 38% of the tested UPEC strains have been found to be colicinogenic;
3. 67% of the colicinogenic UPEC strains were able to lyse all 20 used colicin producer strains, hence these UPEC strains encode and produce either more than one colicin, or a colicin not tested;
4. 19% of the colicinogenic strains produced only ColV, 7% only ColM and 7% only ColE1;
5. plasmids were found in 88% of the colicinogenic strains;
6. 26% of colicinogenic strains harboured conjugative plasmids encoding antibiotic resistance(s) and colicinogenic proprieties and
7. 19% of haemolytic UPEC strains and 44% of non-haemolytic strains were also colicinogenic, 28% of *cnf* encoding strains and 41% of strains not encoding *cnf* were colicinogenic, while 40% of *ibeA* encoding strains and 38% of strains not encoding *ibeA* were colicinogenic.

Acknowledgement

The authors thank B. Bachmann for providing the strain AB1133, A. P. Pugsley for providing the colicinogenic strain collection, D. Ehrlich for strain HB101, R. Schmitt for strains RU4404 and RU4406 and T. J. Silhavy for strain TR51.

Povzetek

V predstavljeni raziskavi smo 110 uropatogenih sevov bakterije *Escherichia coli* (UPEC), ki so jih na Inštitutu za mikrobiologijo in imunologijo Medicinske fakultete v Ljubljani izolirali iz urina bolnikov z urinarno infekcijo, s pomočjo 20 kolicinogenih sevov iz Pugsleyeve zbirke testirali za produkcijo kolicinov. Testiranje je pokazalo, da je bilo 42 sevov UPEC (38%) kolicinogenih. Ker smo testiranje izvedli brez indukcije z mitomicinom C, za katero je znano, da sproži lizogene bakteriofage, lahko predpostavljamo, da dobljeni rezultati temeljijo samo na aktivnosti kolicinov in ne bakteriofagov. Dobljen odstotek kolicinogenih sevov (38%) se ujema tudi z rezultati objavljenimi v podobnih študijah. Vsi kolicinogeni sevi UPEC so povzročili propad producentskih sevov kolicinov ColA, ColB, ColD, ColE2, ColE3, ColE4, ColE5, ColE6, ColE7, ColIa, ColIb, ColK, ColN, MccB17, ColS4, MccC7 in ColE6-J iz Pugsleyeve zbirke. 93% kolicinogenih sevov UPEC je povzročilo propad ColM in ColE1 producentskega seva in 81% kolicinogenih sevov UPEC je povzročilo propad producentskega seva kolicina ColV. 67% kolicinogenih sevov UPEC je povzročilo propad vseh 20 kolicinogenih sevov Pugsleyeve zbirke in 33% kolicinogenih sevov UPEC je povzročilo propad 19 sevov iz Pugsleyeve zbirke. Večina kolicinogenih sevov (67%) sintetizira vsaj dva kolicina, ali pa kolicin, ki ni bil zastopan v Pugsleyevi zbirki kolicinogenih sevov. Kolicine sevov UPEC, ki sintetizirajo samo 1 kolicin, smo prepoznali; 8 sevov UPEC (19%) je sintetiziralo samo ColV, 3 sevi (7%) so sintetizirali samo ColM in 3 sevi (7%) so sintetizirali samo ColE1. Sicer je znano, da različne populacije *E. coli* sintetizirajo različne kombinacije kolicinov, a kombinacija ColV, ColM in ColE1, do sedaj še ni bila ugotovljena. Iz 88% kolicinogenih sevov smo uspeli izolirati plazmidno DNA po metodi alkalne lize. S poskusi konjugacije smo v 11 sevih DL našli konjugativne plazmide z zapisi za odpornost proti antibiotikom in za sintezo kolicinov. Vloga kolicinov v naravnih populacijah še ni popolnoma razjasnjena. Za plazmid ColV je znano, da ima poleg zapisa za kolicin V tudi zapise za virulenčne dejavnike. V naši raziskavi smo ugotovili, da je bilo 19% hemolitičnih sevov UPEC in 44% nehemolitičnih sevov UPEC tudi kolicinogenih; 28% sevov z zapisom *cnf* in 41% sevov brez zapisa *cnf* je bilo kolicinogenih, medtem ko je 40% z zapisom *ibeA* in 38% brez tega zapisa bilo kolicinogenih. Čeprav dobljeni rezultati nakazujejo, da so kolicinogeni sevi manj virulentni kot nekolicinogeni sevi, so za potrditev te domneve potrebne še nadaljnje raziskave.

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ABS ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2006 Vol. 49, Št. 2: 23–32

Sprejeto (accepted): 2007-01-29

Quantitative analysis of the macroinvertebrate community in the river Temenica (SE Slovenia)

Kvantitativna analiza združbe makroinvertebratov v reki Temenici (JV Slovenia)

Mojca PUST, Mihael J. TOMAN

Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, SI-1000 Ljubljana, Slovenia; E-mail: mihael.toman@bf.uni-lj.si.

Abstract. Macroinvertebrate community of the river Temenica was investigated in order to asses the ecological quality of the stream. Different approaches were used in order to compare their usefulness. Surber sampler methodology was used and all together16 quantitative samples were taken at four stream reaches every three months from October 2003 until July 2004. Saprobic and diversity indices showed severe deterioration of water quality longitudinally and a strong negative influence of the town Trebnje on the ecological state of the river Temenica. Multivariate methods DCA and CCA confirmed those results, but also showed greater differences in community structure at sampling site 1 compared to sites 2 and 3 which was not detected using the indices.
Key words: macroinvertebrates, water quality assesment, saprobic index, diversity indices, DCA, CCA

Izvleček. Za oceno ekološke kakovosti reke Temenice smo raziskovali združbo makroinvertebratov. Uporabili smo različne pristope, da bi primerjali njihovo uporabnost. Uporabljena je bila metodologija Surberjevega vzorčevalnika, vzorčili smo na štirih vzorčnih mestih vsake tri mesece od oktobra 2004 do julija 2004, skupno smo pobrali 16 kvantitativnih vzorcev. Saprobni in diverzitetni indeksi so pokazali resno poslabšanje kakovosti vode po toku navzdol in močan negativen vpliv mesta Trebnje na ekološko stanje reke Temenice. Multivariatni metodi DCA in CCA sta potrdili te rezultate, pokazali pa sta tudi večje razlike v strukturi združbe na vzorčnem mestu 1 v primerjavi z mestoma 2 in 3, ki jih z uporabo indeksov nismo zaznali.
Ključne besede: makroinvertebrati, ocena kakovosti vode, saprobni indeks, diverzitetni indeksi, DCA, CCA

Introduction

Macroinvertebrates are extensively used in water quality assessment as they are considered good indicators of environmental pollution. There are various methods for assessing water quality which have different conceptual basis and may therefore provide different information about the aquatic environment. Many authors have compared different methods of summarising responses to pollution (Cao et al. 1996, Rossaro & Pietrangelo 1993). Water quality indices have been developed as a routine technique for water monitoring. Saprobic index is often used as a measure of organic pollution (Urbanič 2004) and is also in use by the Slovenian Ministry of the Environment for biomonitoring of surface waters. Diversity indices are known to be inaccurate at headwater reaches and were also reported to be insensitive to slight and moderate pollution (Cao et al. 1996). However, they can still be used as a complementary method.

Multivariate methods are increasingly used in biological monitoring of water quality (Cao et al. 1996, Rossaro & Pietrangelo 1993). Their main advantage is that they can detect more subtile changes in community structure than the indices. They can also provide information on the responses of taxa to different environmental variables.

The aim of this study was to asses the water quality of the stream Temenica using different methods (saprobic index, different diversity indices and multivariate methods DCA and CCA) and to compare the effectiveness of those methods.

Description of study sites

Our study area was the first part of the disappearing stream Temenica in the south-eastern part of Slovenia. Its length is 25 km and the catchment area comprises 91 km^2.

The upper part of the stream (approx. 5 km) lies in a forested area with little human impact whereas the lowland part with meandering watercourse runs through cultivated land and by the town Trebnje, which is the largest settlement in the area. Elevations range from 560 m at the source to 260 m at the sinking point. Temperatures fluctuate between 2,2–3 ^oC (winter) and 14,2–18,2 ^oC (summer). Stream pH lies within the range 8,1–8,7 and conductivity between 504 and 604 μScm^{-1}. Sampling site 1 was established in the headwaters, site 2 at the beginning of lowland watercourse, and sites 3 and 4 upstream and downstream from Trebnje.

Materials and methods

Macroinvertebrates were sampled on four occasions between October 2003 and July 2004 with 3 month-intervals. At each sampling site 6 random sampling units were taken with a Surber sampler (500 cm^2 sapling area and 0,5 mm mesh size). Several physical and chemical characteristics (Table 1) were measured at the same time.

Table 1: Morphometric and physical characteristics of sampling sites on the river Temenica.
Tabela 1: Morfometrične in fizikalne značilnosti vzorčnih mest v reki Temenici.

Stream reach	Location	Stream order*	Distance from source (km)	Altitude (m)	Mean width (m)	Mean depth (cm)	Maximum temperature (C)
1	Pusti Javor	2	2	360	1,2	6	14,2
2	Stranje pri Velikem Gabru	3	10	300	3,1	31	14,1
3	Štefan	3	18	280	7,3	23	16,9
4	Gorenje Ponikve	3	23	270	5,9	22	18,2

* After Strahler (1952)

All samples were fixed in 4 % formaldehyde in the field. In the laboratory, organisms were sorted, enumerated, identified under a stereomicroscope and stored in 70 % ethyl alcohol. Macroinvertebrates were determined at least to the family level (except groups Hydrachnida and Collembola) using the keys of Bauernfeind & Humpesch (2001), Brinkhurst (1971), Elliot (1977), Gerken & Sternberg (1999), Gloer (2002), Karaman & Pinkster (1977), Nesemann (1997), Reynoldson (1987), Schmedtje & Kohmann (1992), Tachet (2000), Trontelj & Sket (2000), Urbanič et al. (2003), Waringer & Graf (1997), Žiško (2000).

Saprobic index was calculated using saprobic and indicative taxa values after Wegl (1983), Moog (1995) and Urbanič (2004) (Trichoptera). Number of taxa per sampling unit (S) and diversity indices (Shannon-Wiener index $H' = -\Sigma (p_{i*} \ln p_i)$, Eveness index $E = H'/\ln S$ and Simpson's index $D = 1 - \Sigma$

p_i^2) were calculated using PC-ORD (McCune & Mefford 1999). One way ANOVAs were performed (using statistical package SPSS) to determine whether the differences in diversity among sampling sites were significant. Multivariate methods used in this study included detrended correspondence analysis (DCA) and canonical correspondence analysis (CCA). Both were performed using statistical package CANOCO. Data on taxa abundances were log transformed. CCA analysis was performed with reduced data matrix which included only sufficiently common taxa (occurrence in at least 2 samples or at least 4 individuals present) and 5 statistically significant variables (forward selection).

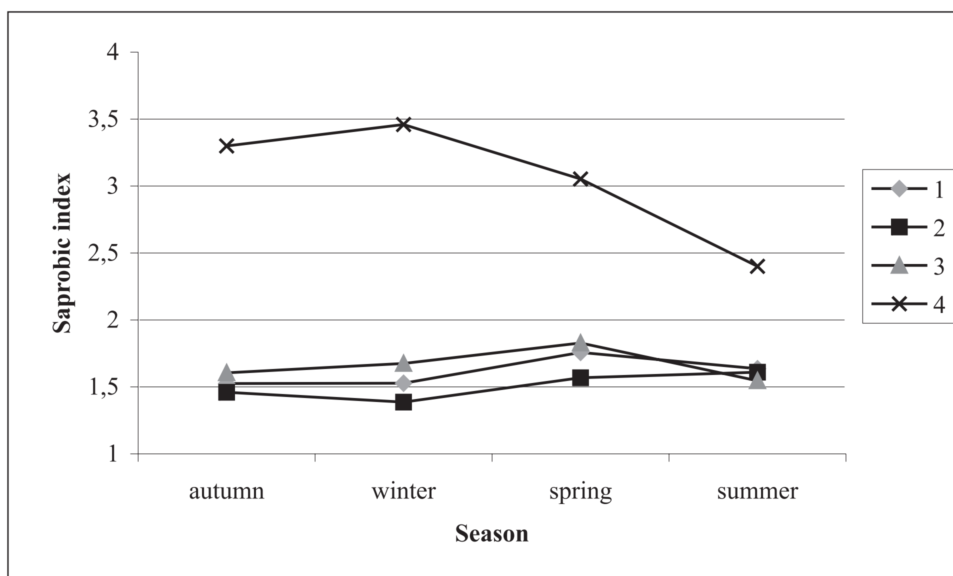

Figure 1: Values of saprobic index at different seasons at four sampling sites of theTemenica river.
Slika 1: Vrednosti saprobnega indeksa v različnih letnih časih na štirih vzorčnih mestih v reki Temenici.

Results

A total of 76550 individuals, representing 120 taxa were collected (Table 2). Saprobic index (Fig. 1) indicates strong organic pollution at sampling site 4 where Tubificidae and Chironomidae were the most abundant. There is an apparent decrease in the value in summertime when Chironomidae (which have a lower saprobic value) outnumbered Tubificidae. Values of the saprobic index at the other three sites were very similar in all seasons and indicate low level of pollution.

Box-plot presentations of taxa number (S) and values of Shannon-Wiener index (H'), Eveness index (E) and Simpson's index (D) are shown in Figure 2. There is a significant decrease in all the values at sampling site 4, which confirms the results of the saprobic index. The number of taxa is also lower at site 1, but diversity indices at this site are equal or even higher (E) than at sites 2 and 3. Using one-way ANOVA we confirmed satistically significant differences in values S, H' and E among sampling sites.

DCA ordination of 16 samples is shown in Figure 3. The first axis explains 40,5 % of the total variance and axis 2 further explains 12,5 %. Samples from each sampling site are clearly separated by the first axis which represents the upstream-downstream gradient and presumably also the pollution gradient. There is a considerable gap between the samples from site 1 and the other samples which indicates significant differences in community structure between the headwaters and the rest of the stream. Axis 2 is apparently related to the seasonal changes in community structure.

Table 2: The abundance of taxa at the sampling sites of Temenica. Abbreviations are added only for taxa used in the CCA

Sampling site		**1**	**2**	**3**	**4**	**Sampling site**		**1**	**2**	**3**	**4**
Taxa	Abbreviation					**Taxa**	Abbreviation				
Polycelis felina	Poly_fel	30	0	0	0	Collembola		3	0	0	0
Dugesia sp.	Duge_sp	0	37	8	178	*Ephemera danica*	Ephe_dan	356	6	2	0
Prostoma sp.	Pros_sp	0	4	0	0	*Ephemerella major*	Ephe_maj	0	14	0	0
Mermithidae	Mer_idae	0	23	3	154	*Ephemerella ignita*	Ephe_ign	2	376	153	17
Holandriana holandri	Hola_hol	3	2232	381	0	*Caenis macrura*		0	0	0	1
Esperiana esperi	Espe_esp	0	8	367	4	*Ecdyonurus sp.*	Ecdy_sp	0	4	1	0
Esperiana daudebartii	Espe_dau	0	0	83	2	*Electrogena sp.*	Elec_sp	49	5	0	0
Theodoxus danubialis	Theo_dan	0	0	174	0	*Rhithrogena sp.*	Rhit_sp	54	32	1	0
Sadleriana fluminensis		0	2	0	0	*Baetis sp.*	Baet_sp	39	712	244	187
Belgrandiella sp.		0	1	0	0	*Centroptilum luteolum*		9	0	0	0
Ancylus fluviatilis	Ancy_flu	0	172	0	3	*Acentrella sinaica*		0	4	0	0
Radix balthica	Radi_bal	0	1	3	3	*Habroleptoides confusa*	Habr_con	35	0	17	1
Physa fontinalis	Phys_fon	0	0	0	12	*Habrophlebia fusca*	Haph_fus	67	0	28	1
Pisidium sp.	Pisi_sp	1	90	65	86	*Habrophlebia lauta*		2	0	0	0
Helobdella stagnalis	Helo_sta	1	0	0	886	*Paraleptophlebia submarginata*	Para_sub	0	0	6	0
Alboglossiphonia hialina		0	0	1	0	*Perlodes sp.*	Perl_sp	20	105	55	2
Glossiphonia concolor	Gloss_con	0	0	0	2	*Isoperla sp*		1	1	0	0
Trocheta bykowskii		0	2	0	0	*Nemoura sp.*	Nemo_sp	33	0	0	0
Dina sp.	Dina_sp	0	0	0	9	*Protonemura sp.*	Prot_sp	16	0	0	0
Eisenella tetraedra	Eisen_tet	2	41	21	7	*Leuctra sp.*	Leuc_sp	9	0	3	0
Lumbriculidae	Lum_idae	0	7	1	0	*Brachyptera sp.*	Brac_sp	20	4	3	0
Stylodrilus heringianus	Styl_her	217	699	249	2219	*Calopteryx virgo*	Calo_vir	0	4	1	0
Tubificidae	Tub_idae	4	143	55	25155	*Calopteryx splendens*		3	10	0	0
Naididae	Nai_idae	2	32	16	44	*Platycnemis pennipes*	Plat_pen	0	0	25	2
Stylaria lacustris	Styl_lac	0	0	123	2	*Lestes viridis*		0	0	0	1
Hydrachnida		1	0	0	0	Lestidae (juv.)		0	0	2	0
Gammarus fossarum	Gamm_fos	296	863	256	6	*Gomphus vulgatissimus*	Gomp_vul	0	22	14	0
Synurella ambulans	Synu_amb	0	0	3	4	*Onychogomphus forcipatus*	Onyc_for	0	11	48	0
Asellus aquaticus	Asel_aqu	1	0	1	47	*Onychogomphus uncatus*	Onyc_unc	0	0	17	0
Austropotamobius torrentium	Aust_tor	0	0	4	0	*Cordulegaster boltoni complex*	Cord_bol	10	0	0	0

Sampling site		**1**	**2**	**3**	**4**
Taxa	**Abbreviation**				
Aphelocheirus aestivalis	Aphe_aes	0	20	85	0
Sialis lutaria		0	0	0	2
Elmis sp.	Elmi_sp	14	398	1361	1
Esolus sp.	Esol_sp	61	4167	1379	18
Limnius sp.	Limn_sp	5	643	131	0
Oulimnius sp.	Ouli_sp	0	346	2474	33
Riolus sp.	Riol_sp	7	65	377	1
Stenelmis sp.		0	0	2	0
Hydraena sp.	Hydn_sp	61	90	145	5
Haliplus sp.		0	0	1	0
Dytiscidae		0	0	1	0
Scirtidae	Sci_idae	276	0	0	0
Eubria sp.		0	1	0	0
Hydropsyche saxonica	Hydr_sax	9	0	0	0
Hydropsyche pellucidula	Hydr_pel	0	71	8	0
Hydropsyche angustipennis	Hydr_ang	0	0	1	13
Hydropsyche siltalai	Hydr_sil	0	11	0	0
Hydropsyche sp. (juv.)	Hydr_sp	0	59	4	0
Silo piceus	Silo_pic	0	571	7	0
Silo pallipes		0	1	0	0
Agapetus delicatulus	Agap_del	0	54	0	0
Glossosoma bifidum		0	1	0	0
Athripsodes cinereus	Athr_cin	0	8	0	0
Athripsodes bilineatus	Athr_bil	0	5	0	0
Leptocerus interruptus	Lept_int	0	0	24	0
Adicella sp.		0	0	2	0
Setodes sp.		0	0	1	0
Mystacides azurea		0	10	0	0
Limnephilus lunatus		0	0	1	2
Chaetopteryx major	Chae_maj	17	0	0	0

Sampling site		**1**	**2**	85	0
Taxa	**Abbreviation**			0	2
Lepidostoma hirtum		0	1	0	0
Potamophylax sp.		0	0	5	0
Odontocerum albicorne		1	0	0	0
Wormaldia subnigra	Worm_sub	0	0	19	0
Wormaldia occipitalis		1	0	0	0
Plectrocnemia sp.		3	0	0	0
Psychomia klapaleki	Psyc_kla	0	5	0	0
Psychomiidae		3	0	0	0
Tinodes rostocki	Tino_ros	7	0	0	0
Lype reducta		1	0	0	0
Ryacophila tristis		2	1	0	0
Ryacophila sp. (s.str.)	Ryac_sp	2	3	2	0
Notidobia ciliaris		0	0	2	0
Hydroptila sp.	Hypt_sp	1	1	26	5
Atherix marginata	Athe_mar	46	147	15	0
Atherix ibis	Athe_ibi	0	6	0	0
Anthomyidae	Ant_idae	0	0	39	1
Chironominae	Chi_nae	22	563	1073	13181
Orthocladinae	Ort_inae	184	292	339	3818
Tanypodinae	Tan_inae	14	23	169	843
Dolichopodidae	Dol_idae	0	1	4	0
Ceratopogonini	Cer_ini	0	7	4	12
Empididae	Emp_idae	*1*	13	25	38
Limoniidae	Lim_idae	64	37	21	2
Psychodidae	Psd_idae	0	1	21	6
Simulium sp.	Simu_sp	54	1568	2128	138
Prosimulium sp.	Prosi_sp	41	0	0	0
Stratiomyidae	Str_idae	2	2	1	0
Tabanidae	Tab_idae	0	15	2	0
Tipulidae		0	1	1	1
Total number of individuals		**2185**	**14875**	**12334**	**47156**
Total number of taxa		**55**	**69**	**73**	**46**

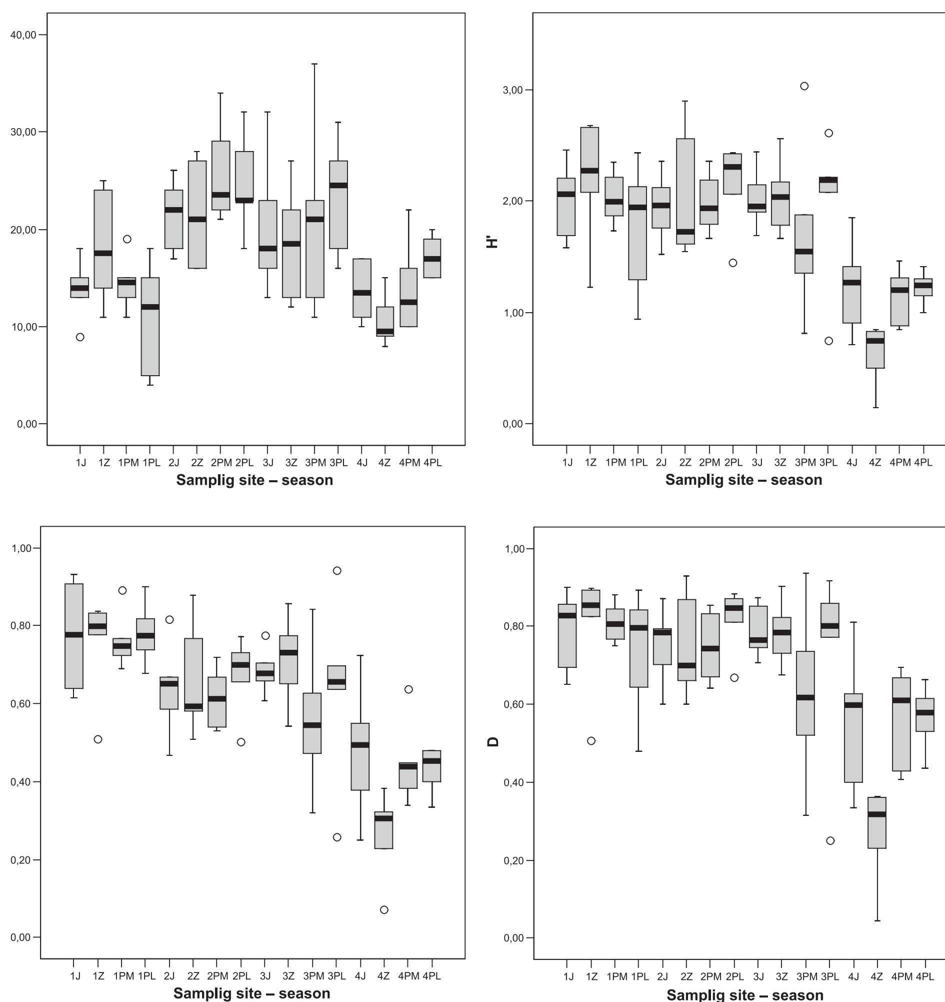

Figure 2: Box-plots of taxa number/sampling unit (S), Shannon-Wiener index (H'), Eveness index (E) and Simpson's index (D) in different seasons at four sampling sites of the Temenica river.

Slika 2: Diagrami kvartilov števila taksonov/vzorčno enoto (S), Shannon-Wienerjevega indeksa (H'), indeksa stalnosti (E) in Simpsonovega indeksa (D) v različnih letnih časih na štirih vzorčnih mestih v reki Temenici.

With 5 selected environmental variables (Fig. 4a) used in the CCA, we explained 64 % of the variance of the taxa-environment relation. Eigenvalues of axes 1 and 2 are 0,38 and 0,28 and display 42 % of the variance. Axis 1 shows high correlation with stream order ($R^2 = –0,95$), and stream width ($R^2 = –0,74$). Taxa which appear on the extreme right side of the biplot (Fig. 4b) therefore prefer headwaters of the stream. Axis 2 is mostly correlated with saprobic index ($R^2 = 0,84$) and may thus present the gradient of organic pollution. Taxa showing affinity (in the upper part of the biplot) to this factor were mostly found at the sampling site 4.

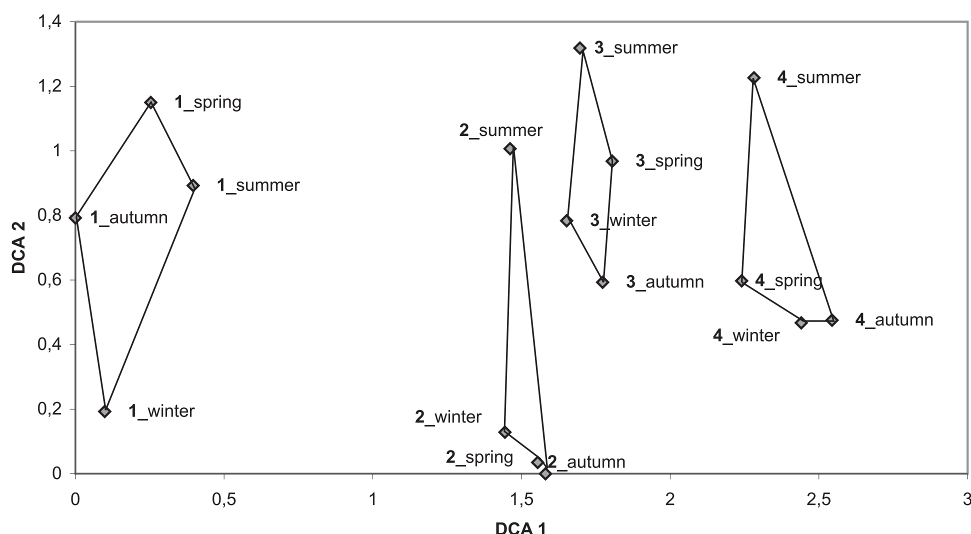

Figure 3: DCA ordination of samples.
Slika 3: DCA ordinacija vzorcev.

Discussion

Macroinvertebrate fauna in the first part of Temenica is relatively diverse with significant differences in community structure among the sampling sites. The fourth site, which lies downstream from Trebnje (population approx. 3000), is especially dissimilar from the rest due to its high saprobic index, low diversity values of indices and the number of taxa. This indicates that the town of Trebnje is a major pollutant of the stream. On the other hand, neither the saprobic index nor the diversity indices showed significant differences among the other three sampling sites.

The values of saprobic index at the fourth sampling site are markedly varying between seasons as a result of changes in abundance of two most abundant groups – Tubificidae (Oligochaeta) and Chironomidae (Diptera). There were also significant seasonal differences in diversity. This indicates the importance of monitoring over a longer period of time.

The first DCA axis can be interpreted both as the upstream-downstream gradient as in the study of Rossaro & Pietrangelo (1993) and the pollution gradient, as suggested by Cao et al. (1996). In comparison with the results of saprobic and diversity indices the samples of the sampling site 1 are here clearly separated from the other samples while the differences in community structure between site 4 and the other sites in much less pronounced than in the case of the indices. The reason for this distribution lies in the small number of individuals and different taxonomic structure at site 1, where the morphometric parameters (depth, width etc.) are very different from those at the other sampling sites. The second DCA axis represents seasonal differences among the samples which are very distinct at all four sampling sites.

Group of taxa, found at sampling site 1 (correlated with stream order), is pointing out in the CCA biplot, as well as the taxa, characteristic for site 4 (correlation with saprobic index). This is in agreement with both the indices and the DCA results. Although indices did not show the difference in community structure between the first and the two next sampling sites, they appear to be better at detecting severe pollution (site 4) than multivariate methods. Complementary use of different methods is therefore the most reasonable approach in water quality assessment.

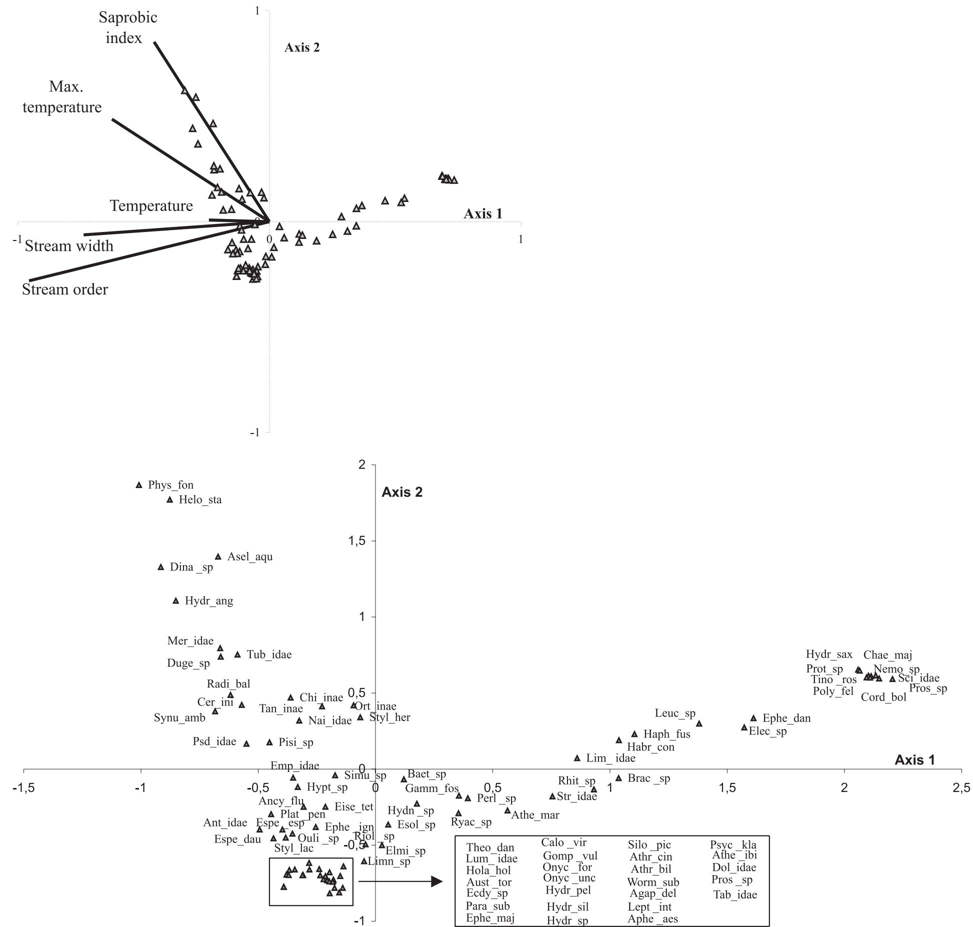

Figure 4: F1 X F2 plane of Canonical correspondence analysis (CCA) between 84 taxa and 5 selected environmental variables. See Table 2 for taxa abbreviations.

Slika 4: F1 x F2 ravnina CCA ordinacijskega diagrama med 84 taksoni in 5 izbranimi okoljkimi spremenljivkami. Glej Tabelo 2 za okrajšave taksonov.

Conclusions

1. High values of saprobic index and low values of diversity indices at sampling site 4 indicate that the town Trebnje as the major pollutant of the Temenica river. Differences among other three sampling sites are not detectable with the use of indices.
2. Axis 1 of DCA biplot, representing the upstream-downstream gradient, revealed great differences in macroinvertebrate community structure between the first and the second sampling site, whereas the distinction of the fourth sampling site is much less pronounced. Seasonal changes in community structure are distinct at all sampling sites.
3. Stream order and saprobic index were the most prominent factors affecting the longitudinal distribution of taxa. Taxa significant for sites 1 and 4 were highly correlated with these two factors.

Povzetek

Makroinvertebrati se kot dobri pokazatelji obremenjevanja okolja veliko uporabljajo pri ocenjevanju kakovosti voda. Metode, ki se pri tem uporabljajo pa temeljijo na različnih principih in lahko dajejo različne informacije o vodnem okolju. Zaradi številnih pomanjkljivosti indeksov, ki se običajno uporabljajo za rutinski biomonitoring celinskih voda, se v vrednotenju kakovosti čedalje pogosteje uporabljajo multivariatne metode. S temi lahko zaznamo manjše spremembe v združbi kot z indeksi, dajo pa nam lahko tudi informacije o odzivu taksonov na posamezne okoljske spremenljivke.

V naši raziskavi smo z analizo združbe makroinvertebratov ocenjevali ekološko stanje prvega dela ponikalne reke Temenice, pri čemer smo uporabili različne metode, da bi preverili njihovo uporabnost pri ocenjevanju kakovosti voda. Kvantitativno vzorčenje s Surberjevim vzorčevalnikom smo izvajali vsake tri mesece od oktobra 2003 do julija 2004 na štirih vzorčnih mestih. Vrednosti saprobnega indeksa, diverzitetnih indeksov ter število taksonov na vzorčno enoto kažejo na močno poslabšanje kakovosti reke Temenice na zadnjem vzorčnem mestu v primerjavi s prvimi tremi in s tem na močan negativen vpliv mesta Trebnje na njeno ekološko stanje. Razvrstitev vzorcev po prvi DCA osi potrjuje našo domnevo o naraščanju obremenjevanja reke po toku navzdol, pri čemer pa četrto vzorčno mesto v primerjavi z rezultati indeksov ni tako izstopajoče. Večje razlike v združbi makroinvertebratov so po tej analizi med prvim vzorčnim mestom in ostalimi tremi. Na vseh vzorčnih mestih so izrazite tudi sezonske razlike v strukturi združbe. Najpomembnejši spremenljivki za razporeditev taksonov sta po rezultatih CCA red vodotoka in vrednost saprobneega indeksa, kar izpostavi taksone, ki so bili najbolj zastopani na prvem in zadnjem vzorčnem mestu.

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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, na 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, predstavljene 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. Wieser 1992: Zur Flora und Fauna des Webersees. Carinthia II 182/102 (1): 159-173.

Trinajstić & 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.

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Toman M. J. 1992: Mikrobiološke značilnosti bioloških čistilnih naprav. Zbornik referatov s posvetovanja DZVS, Gozd Martuljek, pp. 1-7.

12. Format in oblika članka

Članek naj bo poslan v obliki Word dokumenta (doc) ali kot obogateno besedilo (rtf) 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. Uporabljene nomenklaturne vire navedemo v poglavju Metode. Tabele in slike so posebej priložene tekstu. Vse strani (vključno s tabelami in slikami) morajo biti oštevilčene. Glavnemu uredniku je potrebno oddati original, dve kopiji in elektronski zapis na disketi 3,5”, na CD-romu ali kot priponko elektronske pošte (slednjega odda avtor po opravljenih strokovnih in jezikovnih popravkih).

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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 30 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.

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

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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 vo-lume (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 Webersees. Carinthia II 182/102 (1): 159-173.

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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. 1-7.

12. Format and Form of Articles

Articles should be send as Word document (doc) or Rich text format (rtf) 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 in the Methods section. Tables and illustrations shall accompany the texts separately. All pages including tables and figures should be numbered. The original manuscript, two copies, and an electronic copy (after all corrections) on a 3.5” computer diskette, on CD-ROM or by e-mail must be given to the editor-in-chief. All articles must be proofread for professional and language errors before submission.

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