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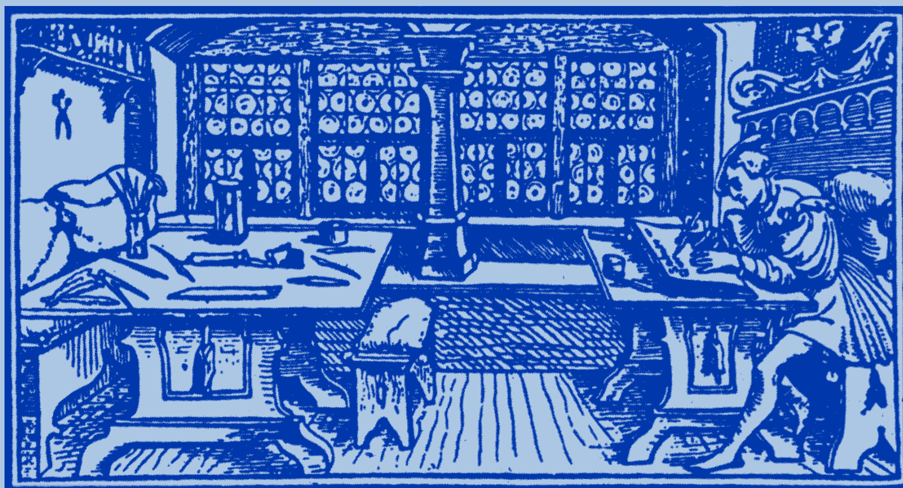

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVII, 2, 2002

MECANISME GENETICE IMPLICATE ÎN DIVERSITATEA LINGVISTICĂ

MANUELA DORDEA* şi NICOLAE COMAN*

SUMMARY. - Genetic Mechanisms Involved in the Linguistic Diversity. The paper is a short review of genetic mechanisms involved in linguistic differentiation. *FOXP2* is the first gene relevant to the human ability to develop language. Due to the polymorphism of nucleotide patterns, it is suggested that gene *FOXP2* could be the target of selection during recent human evolution. Several studies support the hypothesis of a co-evolution of human polymorphism and linguistic differentiation. In this process, migration and isolation are the most important factors involved. Differences in migration patterns of ancient people suggest that, on the long term, language was paternally transmitted. A strong correlation between linguistic and biological diversity is also emphasised. The continuous loss of linguistic and biological diversity can have important consequences for humanity.

O trăsătură specifică omului – *Homo sapiens sapiens* – care-l deosebeşte de restul speciilor animale, este capacitatea sa de comunicare prin limbaj. Particularităţile sociale şi ecologice, în care s-au dezvoltat diferitele grupuri umane în decursul timpului, au dus la moduri diferite de definire, înţelegere şi interpretare a lumii înconjurătoare pe calea limbajului. Rezultatul acestor procese complexe şi dinamice a condus la diversificarea limbilor vorbite şi, implicit, la diversificarea culturală în societatea umană.

Dezvoltarea limbajului articulat se bazează pe un control fin al funcţionării laringelui şi cavităţii bucale, inexistent la cimpanzei sau alte primate.

Recent s-a descoperit prima genă răspunzătoare de capacitatea vorbirii articulate la om, gena *FOXP2* [1]. Această genă, existentă la toate mamiferele, a suferit în cursul evoluţiei o serie de mutaţii, care au condus în linia umană la apariţia limbajului articulat.

Se presupune că modificările acestei gene s-ar fi produs cu 200.000 de ani în urmă, aproximativ în aceeaşi perioadă în care a apărut şi omul modern.

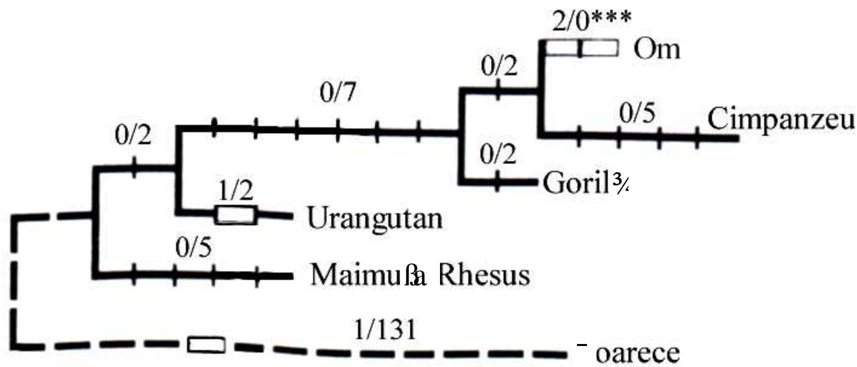
Enard şi colab. [5] au determinat secvenţa genei *FOXP2* la şoarece şi la diferite specii de primate – cimpanzeu, gorilă, urangutan şi maimuţa Rhesus – şi au comparat-o cu secvenţa genei la om. De asemenea, s-a investigat şi variaţia intraspecifică a acestei gene la om.

Gena *FOXP2*, localizată pe cromozomul 7q31, codifică o proteină cu 715 aminoacizi şi se aseamănă cu alte gene reglatoare implicate în dezvoltarea embrionară. Gena aparţine unei clase de factori de transcriere. Proteina codificată conţine o regiune

* *Babeş-Bolyai University, Department of Ecology and Genetics, 3400 Cluj-Napoca, Romania.*
E-mail: ncoman@bioge.ubbcluj.ro

bogată în glutamină, formată din 2 fragmente adiacente de poliglutamină, codificate de un grup de codoni repetitivi CAG şi CAA. Se ştie că asemenea codoni repetitivi au o rată mutaţională ridicată, ceea ce a presupus că gena ar fi putut constitui ţinta acţiunii selecţiei în cursul evoluţiei recente a liniei umane.

De acum 70 milioane de ani, când s-a realizat separarea liniei umane de cea a şoarecilor (Fig. 1), s-au produs 3 modificări în secvenţa proteică, două dintre acestea pe linia filetică umană, după separarea de cimpanzei, cu circa 6 milioane de ani în urmă [5]. Aceste modificări în secvenţa de aminoacizi, rezultatul a 2 substituţii în gena *FOXP2*, au conferit probabil un avantaj selectiv hominidelor purtătoare, deoarece au permis anumite mişcări faciale şi ale cavităţii bucale, esenţiale pentru vorbirea articulată.



F i g . 1 . *Schema arborelui filogenetic al hominidelor faţă de primate şi rozătoare [5].* Barele indică distanţa genetică exprimată prin modificări de nucleotide.

Ipoteza a fost confirmată cu ajutorul testului statistic Tajima, care estimează importanţa presiunii de selecţie asupra unei anumite gene în cursul evoluţiei [2]. Indivizii cu modificări ale genei *FOXP2* au dificultăţi de exprimare şi receptare de limbă, datorită tulburărilor în secvenţa mişcărilor fine orofaciale [18].

Enard şi colab [5] au estimat şi perioada probabilă în care această genă responsabilă pentru vorbirea articulată s-a putut "fixa" în populaţia umană, adică atunci când ambele substituţii s-au transmis la toţi indivizii. Cu o probabilitate de 95%, autorii apreciază că această genă s-a putut răspândi în întreaga populaţie umană nu mai devreme decât acum 120.000 ani.

Desigur este prematur să se afirme cu certitudine că numai gena *FOXP2* ar fi răspunzătoare de apariţia limbajului articulat şi a limbilor vorbite. Ea este însă cu certitudine una dintre ele.

Odată apărută, limba vorbită s-a diversificat în paralel cu diferenţierea genetică (polimorfismul) populaţiei umane, ca rezultat al unei co-evoluţii, în care migraţia şi, respectiv, izolarea a avut un rol hotărâtor.

O serie de studii de genetică populaţională susţin această ipoteză, pornind de la premisa că dacă schimbul de indivizi între populaţii, respectiv fluxul genic, era redus, atunci diferenţierea genetică a populaţiilor care vorbeau limbi diferite se accentuează, comparativ cu a celor care vorbeau aceeaşi limbă.

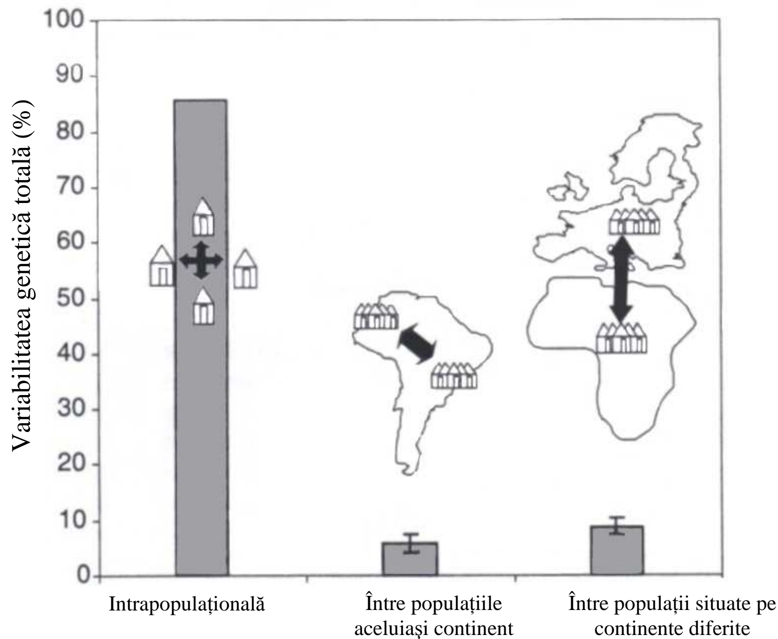
Dupanloup de Ceuninck şi colab. [4] au elaborat o metodă originală care să stabilească dacă barierele lingvistice corespund barierelor genetice dintre populaţii. Metoda s-a bazat pe compararea distanţelor genetice dintre populaţiile aparţinând aceluiaşi grup lingvistic şi a populaţiilor situate de o parte şi de alta a grupului lingvistic evaluat. Folosind programul *AMOVA* de analiză moleculară a varianţei, s-a investigat dacă distribuţia frecvenţelor genice ale diferitelor grupuri lingvistice luate în studiu diferă unele de altele. Metoda s-a aplicat populaţiilor afro-asiatice şi indo-europene, care sunt bine caracterizate prin markeri genetici clasici şi markeri moleculari. Rezultatele lor confirmă ipoteza sincronizării genetice şi lingvistice a celor 2 populaţii umane.

În prezent, studiile de analiză genomică sunt tot mai mult utilizate în explicarea unor probleme demografice, a migraţiei populaţiilor ancestrale şi a diferenţierii lor genetice şi lingvistice. Asemenea analize se bazează pe studiul genomului mitocondrial (*ADNmt*), cu transmitere strict maternă şi a cromozomului sexual Y, cu transmitere strict paternă.

Aşa cum reiese din Fig. 2, *ADNmt* are o variabilitate medie foarte mare între indivizii aceleiaşi populaţii (≈85%). Dacă se compară diversele populaţii ale aceluiaşi continent, variabilitatea *ADNmt* nu este mai mare de 6%, iar între populaţiile diferitelor continente de 9-13% [15]. Variantele cromozomului *Y* sunt mult mai bine localizate geografic, comparativ cu alelele *ADN*-lui mitocondrial sau markerii autosomali, variabilitatea medie fiind de 36%. Mai mult de jumătate din această variabilitate se datorează deosebirilor dintre populaţiile aparţinând la diferite continente.

Diferenţa de variabilitate genetică ar putea fi explicată prin rata mai mare de deplasare, pe distanţe scurte, a femeilor (femeia urmându-şi soţul), care în decursul a sute de generaţii a condus la acumularea variabilităţii *ADNmt* observată în prezent.

Aşa cum am menţionat anterior, analizele de genom pot explica şi căile de migraţie ale unor populaţii ancestrale. Se ştie, de exemplu, că populaţiile de kazahi, uiguri şi kirghizi din Asia Centrală au locuit de-a lungul drumului mătăsii dintre Europa şi Asia, comerţ foarte înfloritor între anii 200 î.C. şi 400 d.C. Analiza secvenţelor de *ADNmt* a acestor populaţii sugerează descendenţa lor din populaţii care se deplasau între Europa şi Asia sau invers, cu peste 2000 de ani în urmă. De asemenea, rezultatele sugerează o deplasare predominantă a femeilor, ceea ce concordă cu organizarea socială existentă în regiune (poligamia). Femeile îşi învăţau copiii limba soţului, ceea ce pe termen lung ar putea semnifica o transmitere a limbajului pe linie paternă. Astfel variaţia genetică a cromozomului *Y*, cel puţin parţial, evoluează în paralel cu diferenţele lingvistice dintre populaţii [15, 17], în timp ce variaţia *ADNmt* nu.



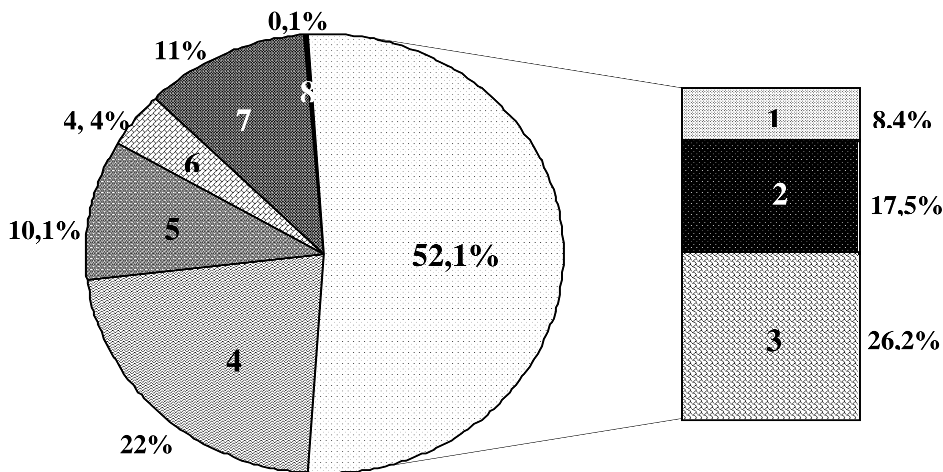
F i g . 2 . *Variabilitatea genetică umană intra- şi interpopulațională (%) [15].*

L i v i n g s t o n e [12] susține că evoluția diversificării lingvistice nu implică în mod obligatoriu o limbă mai funcțională sau un avantaj adaptativ pentru comunitățile ce o utilizau, ci ar fi putut rezulta, mai degrabă ca urmare a unei transmiteri imperfecte de limbă între indivizi. L i v i n g s t o n e [12] susține deci, că diversificarea lingvistică s-ar fi putut realiza, la fel ca şi evoluția lumii vii, conform teoriei neutraliste a lui Kimura (1983), care susține că evoluția poate să se realizeze şi fără acțiunea selecției. Evoluția neutralistă nu poate fi însă singura cauză a diversificării lingvistice, un rol important având şi factorii sociali şi geografici.

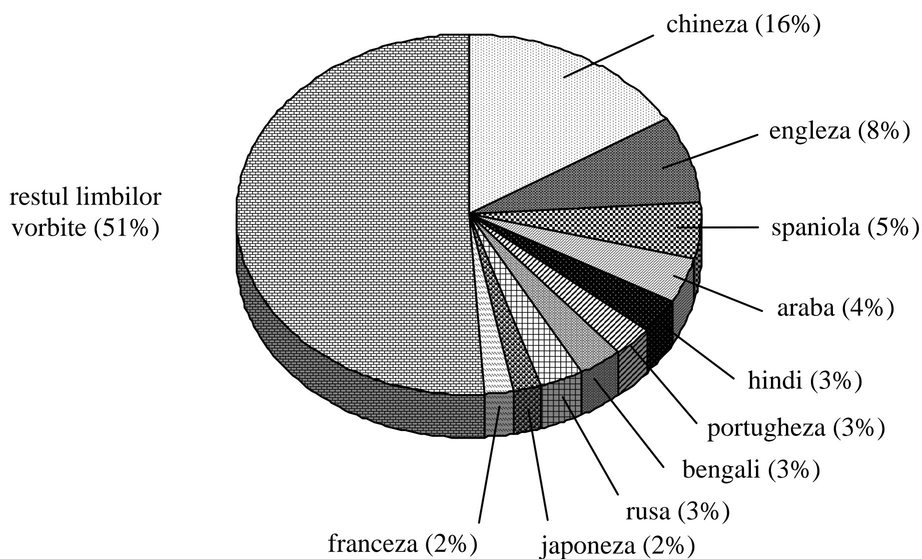
Ce se înțelege prin diversitate lingvistică?

În general, diversitatea lingvistică este dată de numărul diferit de limbi vorbite pe glob.

Ethnologue, cel mai complet catalog privind limbile vorbite pe cele 5 continente, susține că ar exista 6703 limbi (majoritatea orale), din care 32% în Asia, 30% în Africa, 19% în Pacific, 15% în America şi 3% în Europa. Statisticile arată că 52,1% din limbi se vorbesc în comunități cu mai puțin de 10.000 de oameni, iar dintre acestea 17,5% - în comunități cu mai puțin de 1000 de oameni şi 8,4% cu mai puțin de 100 (Fig. 3). Limbile vorbite de comunități de până la 10.000 de oameni totalizează o populație de circa 8 milioane, ceea de reprezintă mai puțin de 0,15% din populația totală estimată pe glob (6 miliarde).



F i g . 3 . *Procentul de limbi vorbite pe glob de comunități de mărimi diferite* (modificat după *[13]*). **1.** 1 – 100 indivizi. **2.** 101 – 1000 indivizi. **3.** 1001 - 10.000 indivizi. **4.** 10.001 – 100.000 indivizi. **5.** 100.001 – 1 milion indivizi. **6.** 1 000 001 - 10 milioane indivizi. **7.** > 10 milioane indivizi. **8.** numai auxiliar (0,4%).



F i g . 4 . *Limbi materne cu cei mai mulți vorbitori: proporție față de populația globului* (modificat după *[13]*).

Rezultă deci, că cea mai mare diversitate lingvistică se întâlneşte în comunităţile mici, cu populaţie indigenă sau minoritară. Din restul de 47,9%, mai puţin de 300 de limbi, cum ar fi chineza, engleza, araba, portugheza, hindi, bengali, rusa şi altele, se vorbesc de către comunităţi cu peste 1 milion de oameni (Fig. 4), însemnând peste 5 miliarde de locuitori de pe glob. Mai mult, aceste limbi se vorbesc doar în câteva ţări de pe glob, majoritatea lor fiind "endemice" pentru ţara respectivă, din care cauză aplicarea unei politici lingvistice naţionale este adeseori dificilă [13].

Harmon [8] stabileşte o listă cu 25 de ţări având o diversitate mare de limbi "endemice" (Tabel 1).

Tabel 1

Primele 25 de ţări de pe glob, după numărul de limbi „endemice"

Nr. crt.	Ţara	Numărul limbilor "endemice"
1	Papua Noua Guinee	847
2	Indonezia	655
3	Nigeria	376
4	India	309
5	Australia	261
6	Mexico	230
7	Cameroon	201
8	Brazilia	185
9	Zair	158
10	Filipine	153
11	USA	143
12	Vanuatu	105
13	Tanzania	101
14	Sudan	97
15	Malaezia	92
16	Etiopia	90
17	China	77
18	Peru	75
19	Chad	74
20	Rusia	71
21	Insulele Solomon	69
22	Nepal	68
23	Columbia	55
24	Coasta de Fildeş	51
25	Canada	47

Limbile vorbite de comunităţi mici, cu populaţie de sub 1000 de oameni, se află sub o permanentă ameninţare de asimilare de către limba majoritară. Se apreciază că limbile pe cale de dispariţie se cifrează între 420 adică 6,3% [6] şi 705 – 10,8% [7]. Unii cercetători sunt şi mai pesimişti, susţinând că în cursul acestui secol 90% din limbile vorbite vor dispărea sau vor fi pe cale să dispară [13].

De altfel, dacă urmărim evoluția istorică, scăderea permanentă a numărului de limbi vorbite este evidentă. Diversitatea lingvistică ar fi avut un maxim la începutul neoliticului (acum circa 10.000 de ani), când se presupune că se vorbeau de 2 ori mai multe limbi decât în prezent [9]. Ulterior, deplasările oamenilor şi expansiunea politică şi economică, chiar înaintea perioadelor de colonizare şi formare de imperii, au contribuit la reducerea diversității lingvistice în diferite părți ale globului, fie prin eliminarea fizică a grupurilor cucerite sau prin asimilare.

Bernard [3] estimează o reducere cu 15% a numărului de limbi vorbite în prezent față de secolul XVI, când a început perioada de colonizare europeană, reducerea fiind mai accentuată în America şi Australia. Din cele 420 de limbi aproape dispărute [6], 138 se vorbesc în Australia şi 67 în America, în special în SUA. La fel ca şi în cazul speciilor biologice, majoritatea limbilor aflate în pericol nu au o documentație sau ea este insuficientă, astfel că dispariția lor va fi totală şi ireversibilă.

Se constată în prezent un fenomen denumit de Phillipson [16] "imperialism lingvistic", răspândirea tot mai mult a limbii engleze pe glob şi invadarea altor limbi cu termeni de origine engleză. După Kachru [10], vorbitorii de limbă engleză ca limbă maternă, limbă oficială sau limbă străină, se cifrează între 700 milioane şi 2 miliarde. Numai în Asia, populația vorbitoare de limbă engleză este de circa 350 milioane [11].

Luând în considerare faptul că evoluția lingvistică s-a realizat în paralel cu evoluția lumii vii, Harmon [8] a făcut o comparație interesantă între țările cu diversitate foarte mare (privind speciile de vertebrate, insecte şi plante superioare) conform listei IUCN (International Union for the Conservation of Nature) şi țările cu cele mai numeroase limbi "endemice". A constatat că 10 din 12 țări cu diversitate biologică foarte mare (83%) figurează printre primele 25 de țări cu limbi "endemice" numeroase. A constatat şi excepții, cum ar fi Papua Noua Guinee, țara cu diversitatea lingvistică cea mai mare (847 limbi vorbite), dar cu diversitate biologică redusă.

Acest paralelism între diversitatea biologică şi cea lingvistică capătă tot mai mult contur, ceea ce impune o abordare holistă a problemei diversității. Limba vorbită are un rol cheie în relația om – natură, fiind forma codificată prin care omul transmite cunoştințe legate de mediul în care trăieşte şi evoluează. În acest sens sunt edificatoare cuvintele lui Mühlhäusler [14]: "Life in a particular human environment is dependent on people's ability to talk about it". (Viața într-un anumit mediu depinde de capacitatea omului de a vorbi despre acesta).

Scăderea continuă a diversității lingvistice, culturale şi biologice va avea fără îndoială consecințe serioase asupra umanității şi a întregii planete.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVII, 2, 2002

ADDITIONAL TAXA WITHIN THE SILICEOUS MICROFLORISTIC ASSEMBLAGES FROM THE BÂRZĂVIŢA II QUARRY (ARAD COUNTY, ROMANIA)

OVIDIU BARBU* and VLAD CODREA*

SUMMARY .- The deposits of the lower tuffaceous-diatomaceous complex, studied on a log from the Bârzăviţa II quarry, contain a rich assemblage of siliceous microorganisms including: diatoms, silicoflagellates, ebridians, sponge spicules, dinoflagellates, chrysomonadines and phytolites. The aim of this study was to report the presence of three additional taxa within the associations: one belonging to ebridians (*Podamphora* sp.) and the others to dinoflagellates (*Carduifolia gracilis* Hovasse and *Calicipedinium quadripes* Dumitrică). Two of these taxa (*Podamphora* and *Carduifolia*) were reported a long time ago from the Zărand Basin, but not from the Bârzăviţa site. Therefore, this discovery brings them to interest again. The third taxon (*Calicipedinium*) is a novelty, for both the Bârzăviţa II quarry and the whole sedimentary Middle Miocene Zărand Basin.

Due to its specific features**,** the Neogene Zărand Basin takes a special place among the "gulf-basins" from the western side of the Apuseni Mountains. A very peculiar sedimentation took place during the Early Sarmatian (*i.e.*, Volhynian *sensu* Suess), when a thick pile of tuffs and diatomites were accumulated in this area. The diatomites are still exploited in a quarry named Bârzăviţa II**,** located on the left bank of Bârzăviţa Valley, very close to the Minişul de Sus village, Arad county.

The study of the succession cropping out in this quarry showed that the Lower Sarmatian "lower tuffaceous-diatomaceous complex" [5] lies unconformly on the older basin's basement [2]. This one is represented by the Permo-Werfenian belonging to the main component of the Codru Nappe System, the Finiş-Gârda Nappe [6]. The Sarmatian includes an approximately 50 m thick succession, consisting of lamellar diatomite interbedded with partially altered tuffs and lapillistones. The top of the log corresponds to a flow plate of andesitic lava, which preserved against erosion the softer rocks located below [3].

The study of the samples collected from this site revealed a rich assemblage of siliceous microorganisms including diatoms, silicoflagellates, ebridians, sponge spicules, dinoflagellates, chrysomonadines and phytolites. Within these assemblages, we found – for the first time in this area – several taxa belonging to ebridians and dinoflagellates.

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* *Babeş-Bolyai University, Department of Geology, Kogălniceanu str.,1, 3400 Cluj-Napoca, Romania. E-mail: obarbu@bioge.ubbcluj.ro*

Paleontology.

OPALOZOA
Ebriophycea Loeblich III 1970
Ebriales Hanigberg *et al.* 1964
Heremesinaceae Hovasse 1943

Podamphora sp.

Description. Siliceous skeleton, including two parts: **i**. a rectangular basal one, with actines separating a series of upper and lower windows; **ii**. a chamber-like second one, located at the nuclear pole – *lorica* – with a reticular ornamentation and an opening at the end of a short neck. Fine ellipsoid pores bore through the basal part of the lorica.

Discussion. The *lorica,* present on the normal skeleton of certain fossil specimens (*Ebriopsis, Hovassebria, Podamphora* a.s.o.) has been considered for a long time as being an allogromiid foraminifera, using the ebridian skeleton as a foreign matter agglutinated to its shell. However, this fact cannot be accepted since the present-day allogromiids rarely occur in seawater and do not secrete silicon. The ebridian skeleton always had the *lorica* at the nuclear pole.

The representatives of the *Podamphora* genus are documented between the Paleocene-Miocene. Up to the present, this taxon has been reported in this Miocene basin only from the Cărand site: *Podamphora elgeir* Gemeinhardt 1931 [7]. Therefore, our discovery from Bârzăvița II quarry shows that its distribution area can be extended to other Zărand Basin areas as well.

PYROPHYTA

Dinophyceae Pascher 1914
Gymnodinida Schutt 1896
Actiniscidae Kützing 1849
Carduifolia Hovasse 1932

Carduifolia gracilis Hovasse 1932
Plate 1; Figs. 1 and 2

Description. The skeleton consists of a central apical body and four tricostate feet descending – two on each side – from its extremities. The feet have lateral crests and longitudinal furrows on the convex side.

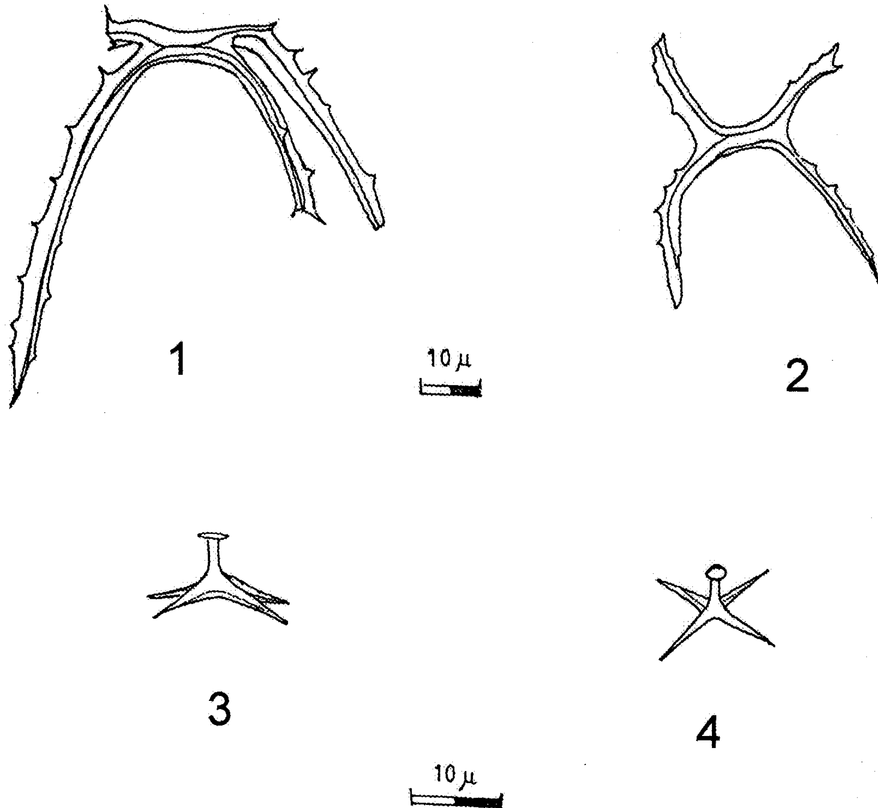
Discussion. In our country, D u m i t r i c ă [4] reported this taxon from the Middle Miocene from Păuşeşti-Otăsău (Romania) and DSDP (Deep Sea Drilling Project) 206 in the Southwestern Pacific.

Calicipedinium Dumitrică 1973

Calicipedinium quadripes Dumitrică 1973
Plate 1; Figs. 3 and 4

Description. Candlestick-shaped massive siliceous spicule, consisting of an axial body with a cup-like plate at the apical end and four tricostate arms at the basal end. The arms get narrower towards their extremities and are disposed in a star-like outline.

Discussion. D u m i t r i c ă [4] described this taxon from the Middle Miocene so-called „Radiolarian Schist Horizon", at Păuşeşti-Otăsău and Chiojdeanca (Romania). This author showed that this genus had close morphological affinities with the *Actiniscus*. This taxon has not yet recorded from the Zărand Basin.



P l a t e 1. *New microfloristic taxa recorded from the Bârzăvița II quarry.*
Figs. 1 and 2. Carduifolia gracilis *Hovasse 1932*.
Figs. 3 and 4. Calicipedinium quadripes *Dumitrică 1973*.

Conclusions. 1. This study reports on three additional new taxa within the siliceous microorganism assemblages from the Zărand Basin, at Bârzăvița II quarry. One of these taxa belongs to ebridians (*Podamphora*), the others two to dinoflagellates (*Carduifolia gracilis* and *Calicipedinium quadripes*). *Podamphora* and *Carduifolia* had been reported long time ago from the Zărand Basin, but not from the Bârzăvița II site. *Calicipedinium* represents a novelty for both the Bârzăvița II quarry and the whole sedimentary basin.

2. The sedimentation of the "tuffaceous-diatomaceous complex" took place in a dominance of the freshwater environment. However, the presence of these taxa, corroborated with data supplied by the complete siliceous microorganism assemblages [1], makes evident the existence of several short marine–brackish events at different levels within the Bârzăvița II quarry succession. These events may be related to some ingressions originating from the open Pannonian Basin realm, or to a close environment, with high evaporation episodes and low freshwater input carried on by the rivers.

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LA PRÉSENCE DE *CYCLOPS PRAEALPINUS* KIEFER, 1939 (CRUSTACEA, COPEPODA) EN ROUMANIE

CORNELIU PLEŞA* et SANDA IEPURE*

SUMMARY. – **The Presence of *Cyclops praealpinus* Kiefer, 1939 (Crustacea, Copepoda) in Romania**. *Cyclops praealpinus (*Cyclopoida) is reported from the alpine lakes in Retezat Mountains (Western Meridional Carpathians). *C. prealpinus* Kiefer, which has *terra typica* in Lake Konstanz (South Germany) is for the first time recorded from Romania. Seven lakes were sampled; the detailed taxonomic study was done on the material from one of these lakes, the Gemenele Lake, to establish the validity of this species contested by some authors.

Par suite d'une étude détaillée des populations de *Cyclopides* prélevées du Lac Constance (Bodensee) du sud de l'Allemagne et que F i s c h e r [3][1] avait considérées comme appartenant à la très répandue espèce „*Cyclops strenuus*", K i e f e r [4] sépare une partie des formes examinées sous le nom de *Cyclops praealpinus*, en se basant sur des arguments d'ordre qualitatif et quantitatif.

Dans la présente Note, on donne une re-description détaillée de *Cyclops praealpinus*, identifié dans le riche matériel qu'un collectif de chercheurs de la Station Zoologique de Sinaia, dirigé par M. le Dr. Constantin C i u b u c , a collecté dans plusieurs lacs du massif de Retezat par des prises planctoniques.

Les premières données concernant la faune des lacs du massif du Retezat (Carpates Méridionaux) sont dues à S z i l á d y [9], qui signale deux espèces de Cyclopides, dont une est le „*Cyclops strenuus*" de Fischer. Jusqu'à nos jours, il semble que personne n'a pas signalé d'autres Cyclopides dans les lacs alpins de ce massif.

Matériel et méthodes. Les lacs du massif de Retezat d'où provient le matériel étudié sont les suivants:

Tăul Ştirbu, 3 août, 29 septembre;
Tăul Caprelor, 3 août;
Tăul Porţii, 30 septembre;
Le lac Judele, 30 août;
Le lac de Stânişoara, 6 août;
Tăul Negru, 11 juin, 5 août, 28 septembre;
Le lac de Gemenele, 5 et 10 juin, 1 août, 27 septembre.

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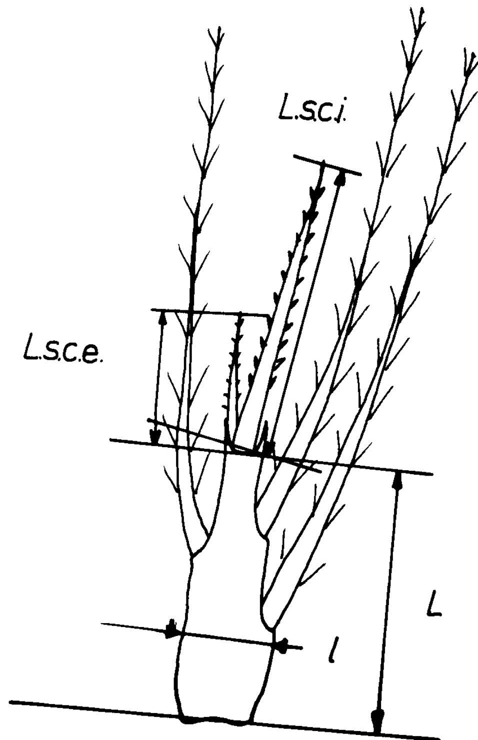
* *Institut de Spéologie «Emile Racovitza» de Cluj, Roumanie. E-mail: plesa@mailexcite.com, siepure@hasdeu.ubbcluj.ro*

[1] L i n d b e r g [6] avait établi que le nom correct de cette espèce serait *Cyclops rubens* (Jurine, 1820).

Toutes les prises ont été prélevées en 2001. Les résultats des mensurations effectuées sur des individus provenant du lac de Gemenele sont présentés dans les Tableaux 1 et 2.

La technique de mensuration que nous avons utilisée dans le cas du dernier article de l'endopodite P4 est illustrée dans la Fig. 1.



F i g . 1. *Mesurements sur l'endopodite 3 de la P4.*

Description du taxon.

Femelle. Aspect général cyclopoide (Fig. 2 B), avec un rostre plus ou moins visible, selon l'état de contraction de l'animal. Longueur (sans les soies furcales) comprise entre 1.173 et 1.507 μ et largeur (céphalon), entre 388 et 469 μ. Les rapports longueur/largeur au niveau du céphalothorax sont indiqués dans le Tableau 1.

Antennules (A1) composées de 17 articles, parfois mal différenciés du fait que les lignes de séparation sont incertaines surtout entre les articles 7-13. Rabattues, les antennules arrivent jusqu'à la moitié du 2-ème segment thoracique. Le nombre et la disposition des soies qui ornent chaque article sont illustrées dans la Fig. 2 C. Chez les femelles que nous avons examinées il n'existait pas des appendices semblables aux „aesthetascs".

Tableau 1

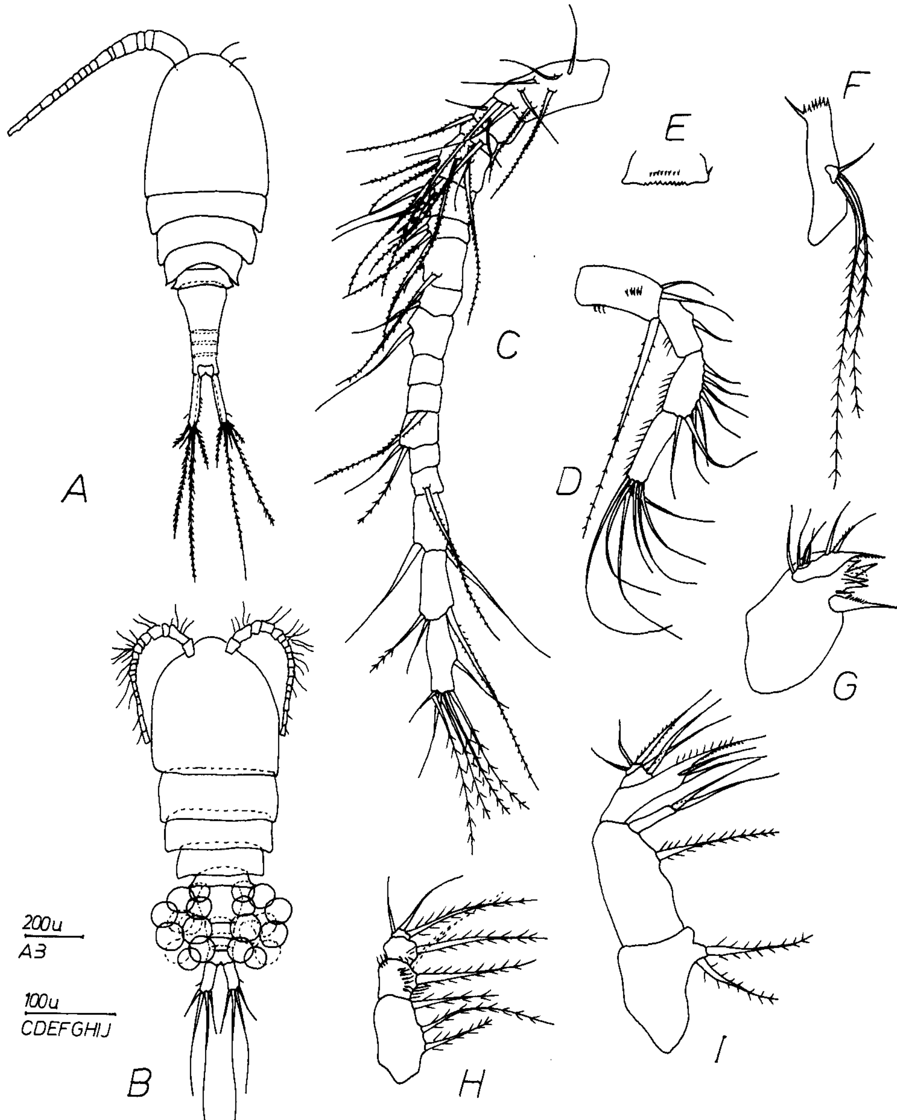
Dimensions (en microns) mesurées sur des femelles de *Cyclops praealpinus* Kiefer

	CORPS					ENDOPODITE 3 DE LA P4							BRANCHES FURCALES					
Nº préparation	Longueur totale	Longueur céphalothorax	Longueur abdomen	Largeur céphalothorax	Rapport L. céphaloth. / L. abdomen	Longueur article	Largeur article	Rapport longueur / largeur article	Longueur épine apicale intérieure	Longueur épine apicale extérieure	Rapport L. ép. apic. int./ L. article	Rapport L. ép. apic. int./ L. ép. apic. ext.	Longueur	Largeur	Rapport longueur/ largeur	L. soie dorsale proximale	L. soie dorsale distale	Longueurs des appendices apicaux
13	1.173	686	487	460	1,41	100	35	2,86	133	46	1,33	2.89	140	32	4.38	40	102	99;397; 478;208
3	1.245	821	424	415	1,94	80	34	2,35	107	42	1,34	2,55	113	32	3,54	34	117	86;361; 469;181
9	1.245	749	496	388	1,51	96	35	2,74	100	46	1,04	2,17	128	31	4,13	42	87	90;307;3 97;171
1	1.263	812	451	448	1,80	97	33	3,03	115	52	1,19	2,21	126	32	3,94	32	80	145;370; 478;190
15	1.272	821	451	469	1,82	91	35	2,60	112	45	1,23	2,49	126	23	5,48	25	88	99;406; 469;194
14	1.299	803	496	460	1,62	98	32	3,06	108	45	1,10	2,40	115	36	3,19	32	90	104;388; 487;185
4	1.344	794	550	469	1,44	82	32	2,56	105	42	1,28	2,50	135	32	4,22	42	100	102;380; 460;199
7	1.380	920	469	442	1,96	96	37	2,59	115	47	1,20	2,45	135	35	3,86	37	82	108;343; 460;162
11	1.386	926	460	424	2,01	90	37	2,43	110	48	1,22	2,29	126	31	4,06	37	95	90;361; 397;180
10	1.406	911	495	469	1,84	97	36	2,69	107	35	1,10	3,06	131	35	3,74	41	87	99;406; 496;199
8	1.434	965	469	460	2,06	110	27	4,07	112	55	1,02	2,04	131	35	3,74	40	72	107;397; 487;199
2	1.452	938	514	433	1,82	106	32	3,31	117	53	1,10	2,21	145	36	4,03	30	90	117;325; 469;208
6	1.470	902	568	451	1,59	95	36	2,64	106	50	1,16	2,12	137	35	3,91	37	78	110;406; 487;208
12	1.507	957	550	463	1,74	98	37	2,65	112	50	1,14	2,24	142	31	4,58	41	95	104;406; 505;210

Antennes (A2) composées de 4 articles (Fig. 2 D). Chez certains exemplaires, les soies du 3-ème article sont insères sur de petites saillies à aspect de scie.

Pièces buccales. Labium mince (Fig. 2 E), mandibule pourvue d'une palpe mandibulaire très longue, composée d'un petit article sur lequel s'insèrent 3 soies de longueur différente (Fig. 2 F). Maxillule petite et trapue (Fig. 2 G). Maxille (Fig. 2 I) composée de 5 articles distincts, dont le 2-ème, à l'insertion apicale, est pourvu d’un autre article allongé, portant à son extrémité deux soies effilées. L'article basal de la maxille présent vers sa partie distale un double gonflement, dont un porte deux appendices ayant la forme de soies. Le dernier article de la maxille a l’aspect caractéristique d'une „tourelle" armée d'un faisceau composé de 3 soies. Maxillipède

(Fig. 2 H) composé de 4 articles bien distincts. Le basal est armé de 3 appendices, dont celui central est le plus long et le 3-ème a une insertion apicale. Le 2-ème article du maxillipède avec 2 appendices, toujours en forme de soies, portant sur leur rebord une courte rangée d'épinules. Le 3-ème article est pourvu d'une seule soie longue, et l'article apical, de 3 appendices de forme et de longueur différentes.



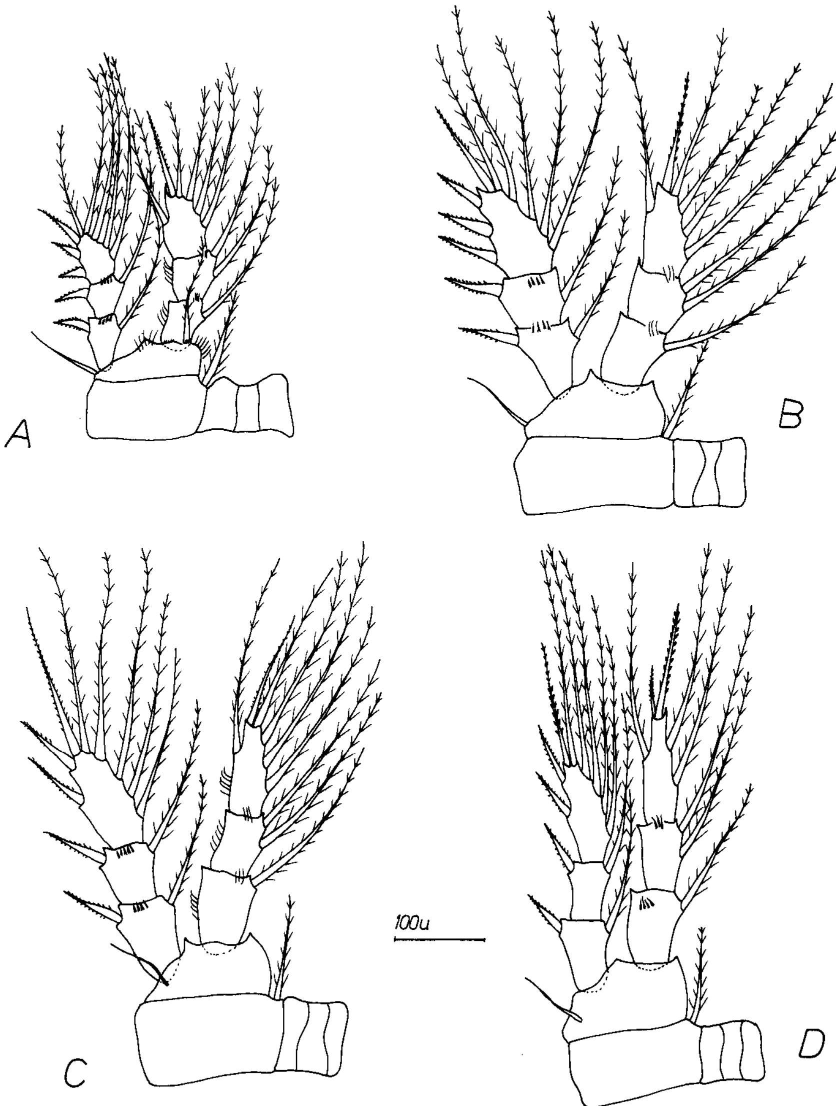
F i g . 2. Cyclops praealpinus *Kiefer, femelle*.
A – Aspect général (selon Kiefer). B – Aspect général (orig.). C – Antennule. D – Antenne. E – Labium. F – Mandibule. G – Maxillule. H – Maxillipede. I – Maxille.

Pattes natatoires P1-P4. Leur structure est illustrée dans les Fig. 3 A - D. Formule des épines 3.4.3.3, celle des soies 5.5.5.5. Lamelles hyalines dépourvues d'ornementations (Fig. 4 F). Pour le dernier article de l'endopodite P4, les rapports dimensionnels les plus importants varient entre les limites suivantes (Tableau 1):

– longueur / largeur de l'article = 2,35-4,07;

– longueur de l'épine apicale interne / longueur de l'article = 1,02-1,34;

– longueur de l'épine apicale interne / longueur de l'épine apicale externe = 2,04-3,06.



F i g . 3. Cyclops praealpinus Kiefer, femelle. A – P1. B – P2. C – P3. D – P4.

Tableau 2

Dimensions (en microns) mesurées sur des mâles de *Cyclops praealpinus* Kiefer

	CORPS					ENDOPODITE 3 DE LA P4							BRANCHES FURCALES					
N° préparation	Longueur totale	Longueur céphalothorax	Longueur abdomen	Largeur céphalothorax	Rapport L. céphaloth. / L. abdomen	Longueur article	Largeur article	Rapport longueur / largeur article	Longueur épine apicale intérieure	Longueur épine apicale extérieure	Rapport L. ép. apic. int./ L. article	Rapport L. ép. apic. int./ L. ép. apic. ext.	Longueur	Largeur	Rapport longueur/ largeur	L. soie dorsale proximale	L. soie dorsale distale	Longueurs des appendices apicaux
6	911	541	370	334	1,46	77	27	2,85	95	46	1,23	2,07	93	28	3,32	33	107	82; ? ; ? ;82
12	957	578	379	343	1,53	81	27	3,00	96	42	1,19	2,29	104	27	3,85	25	107	102;307; 370;149
11	975	659	316	289	2,09	72	27	2,67	112	27	1,57	4,15	75	27	2,78	30	77	75;280; 361;126
8	984	578	406	316	1.42	87	26	3,35	120	43	1,38	2,79	100	27	3,70	32	?	90;298; 325;172
7	992	568	424	343	1,34	80	30	26,7	87	43	1,09	2,02	90	29	3,10	35	80	87;289; 334;90
9	1.002	650	352	316	1,85	77	28	2,75	85	41	1,10	2,07	92	28	3,29	32	95	95;271; 352;163
5	1.013	668	345	316	1,94	82	27	3,04	90	42	1,10	2,14	90	28	3,21	27	87	70;289; 370;130
10	1.111	695	416	316	1,67	84	25	3,36	92	30	1,09	3,07	87	27	3,22	30	78	95;317; 379;154
4	1.119	740	379	307	1,95	80	27	2,96	95	37	1,19	2,57	87	27	3,22	33	92	90;289; 352;167
3	1.124	749	375	302	2,00	80	27	2,96	85	35	1,06	2,43	97	28	3,46	35	86	90;290; 334;158
2	1.141	780	361	334	2,16	83	27	3,07	87	35	1,05	2,49	90	32	2,81	34	126	90;298; 343;158

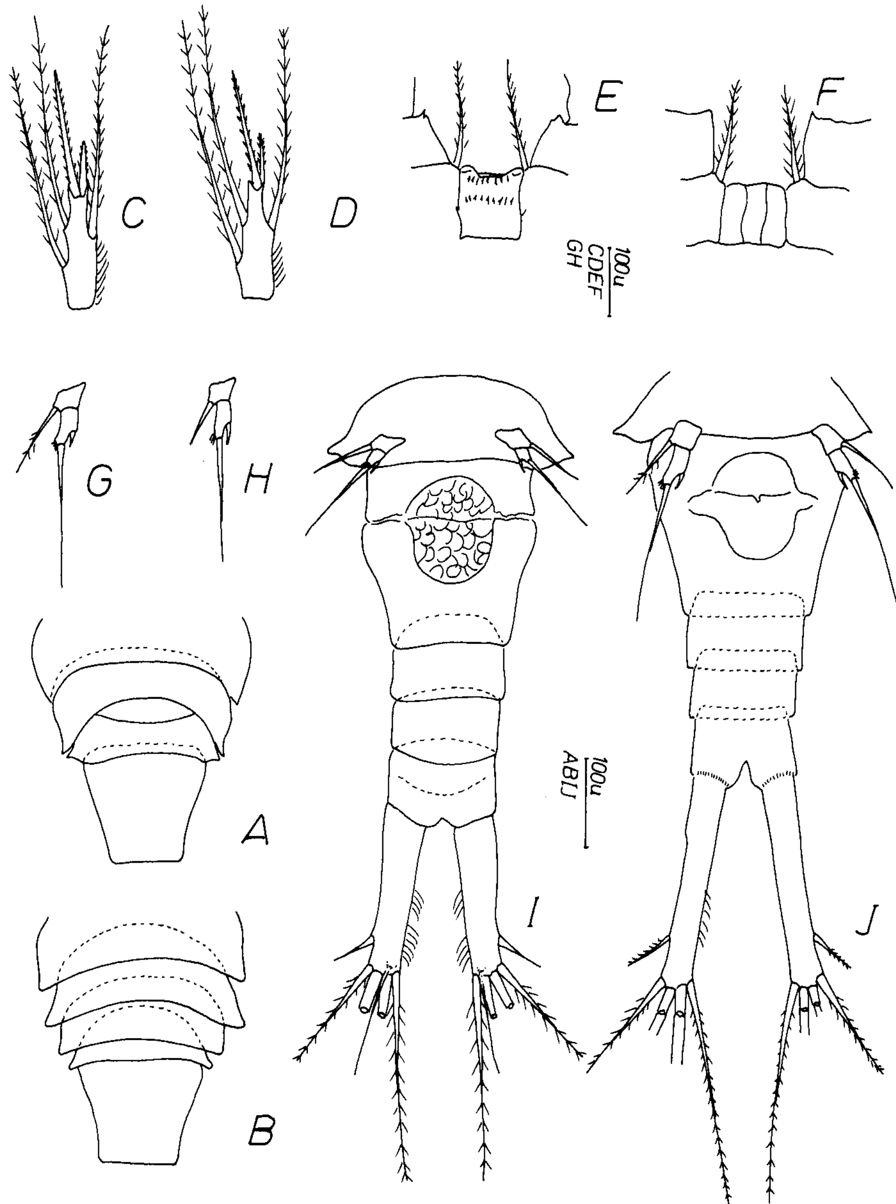
Patte P5 composée de 2 articles, ayant la même forme que celle connue chez tous les représentants du genre *Cyclops*. La petite épine insérée au centre de la face interne du 2-ème article est mince (Fig. 4 H), et la soie apicale est environ 6 fois plus longue que cette épine.

Céphalothorax à 5-ème segment normalement conformé (Fig. 5 B), avec la protubérance latérale de forme habituelle.

Abdomen. Segment génital environ aussi long que large, avec un réceptacle séminal circulaire typique (Fig. 4 I). Les segments abdominaux 2, 3 et 4 de même aussi longs que larges, sans aucune ornementation latérale ou terminale, à l'exception du dernier, qui porte sur le rebord ventral, de chaque côté, une rangée d'épinules.

Opercule anal circulaire et glabre.

Branches furcales légèrement divergentes. Le rapport longueur / largeur varie de 3,19 à 5,48 / 1. Leur rebord interne est poilu, avec de longues soies distancées et inégales (Fig. 4 I). Le rapport entre la longueur des appendices apicaux des branches furcales est donné dans le Tableau 1.



F i g . 4. Cyclops praealpinus *Kiefer, femelle.*
A – Partie distale du céphalothorax (selon Kiefer). B – Partie distale du céphalothorax (orig.). C – Endopodite 3 de la P4 (selon Kiefer). D – Endopodite 3 de la P4 (orig.). E – Lamelle hyaline des pattes natatoires (selon Kiefer). F – Lamelle hyaline (orig.).G – P5 (selon Kiefer). H – P5 (orig.).I – Abdomen, vue ventrale (orig.). J – Abdomen, vue ventrale (selon Kiefer). K – Branches furcales (selon Kiefer).

La ligne saillante dorsale si caractéristique pour les représentants du genre *Cyclops*. est très faiblement marquée, parfois même absente.

Sacs ovigères contenant 12-14 œufs, de 112-126 μ de diamètre. On a observé aussi des spermatophores attachés à l'orifice génital de la femelle (Fig. 5 C).

Mâle. Plus petit et plus svelte que la femelle. Longueur totale (moins les soies furcales) comprise entre 911 et 1.141 μ, largeur (céphalon) entre 289 et 343 μ (Tableau 2).

Antennule préhensile. Le nombre d'articles qui la composent ne peut que très difficilement être établi (12, 16 ou même 18) (Fig. 5 A). On n'a pas remarqué des „aesthetascs" sur les articles antennaires.

Antennes (A2) composée de 4 articles ayant la même structure que chez la femelle.

Pattes natatoires P1-P4 avec la même formule des épines et des soies que chez la femelle, exceptionnellement 3.4.4.3. Le domaine de variation des principaux rapports dimensionnels pour l'endopodite P4 est (Tableau 2):

– longueur / largeur du dernier article = 2,67-3,36;

–longueur de l'épine apicale interne / longueur de l'article = 1,05-1,56;

– longueur de l'épine apicale interne / longueur de l'épine apicale externe = 2,02-4,15.

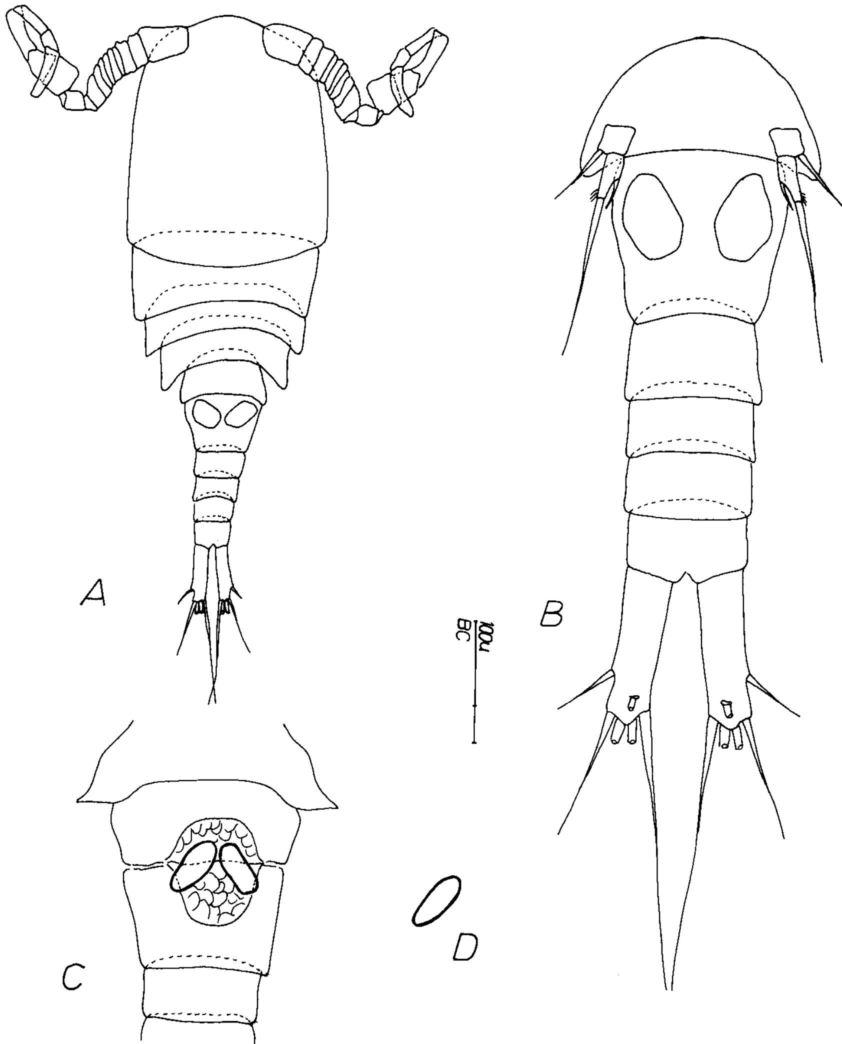
P 5 (Fig. 5 B) comme chez la femelle, mais avec l'épine interne du 2-ème article plus élancé.

P 6 très bien marqué, armé de 2 soies de longueur inégale. Le segment anal présente sur le rebord distal de sa face ventrale une rangée d'épinules fines.

Branches furcales légèrement divergentes (Fig. 5 B), avec les rebords internes glabres. Le rapport longueur / largeur varie de 2,78 à 3,85 / 1.

Remarques taxonomiques et discussions. Afin de permettre une comparaison des données présentées ci-dessus avec celles extraites de la description originale de Kiefer [4], nous avons réuni les figures données par ce dernier (Fig. 2 A et Fig. 4 A, C, E, G, J, K). En effectuant des mensurations sur ses dessins, on constate que les femelles qu'il a étudiées ont une longueur de 1.405-1.635 μ, tandis que nos exemplaires ont des longueurs comprises entre 1.173 et 1.507 μ. La cause pourrait en être le fait que les populations comparées proviennent de différents biotopes: le grand lac de Constance, situé à une basse altitude, et les petits lacs alpins du massif de Retezat, situés beaucoup plus haut. On peut constater aussi une petite différence quant à la longueur des branches furcales, mais à notre avis celle-ci tient à la variabilité individuelle.

En ce qui concerne la „carène" qui devrait être présente sur la branche furcale, elle est très peu évidente, tel que Kiefer l'a déjà remarqué à juste titre dans sa description originale: „*…Die dorsale Chitinlängsleiste auf jedem Furkalast ist nich besonders stark ausgebildet*" [4, p. 99]. On constate aussi que les lamelles hyalines de pattes natatoires de nos exemplaires sont dépourvues d'épinules (Fig. 4 F), tandis que celles-ci sont présentes chez les exemplaires examinés par Kiefer (Fig. 4 E).



F i g . 5. Cyclops praealpinus *Kiefer.*
A – Mâle, aspect général. B. – Abdomen du mâle, vue ventrale. C. – Segment génital de la femelle, avec les spermatophores fixés D. – Spermatophore isolé.

Quoiqu'il en soit, les points de vue exprimés ultérieurement par divers auteurs à l'égard de la validité taxonomique de l'espèce décrite par K i e f e r ne coïncident pas. L'auteur même [5] fini par mettre *Cyclops praealpinus* en synonymie avec *C. abyssorum* Sars, 1863, en le considérant comme une sous-espèce de celui-ci. D'autre part, Š r á m e k - H u š e k [8] confirme la validité de l'espèce, et D u s s a r t [1] la reconnaît également, en créant même des sous-espèces. L'opinion de K i e f e r a été reprise par M o n c h e n k o [7], et plus récemment par E i n s l e [2].

En conclusion, la validité taxonomique de *C. praealpinus* semble toujours être sujette à caution pour certains auteurs, à notre avis le problème ne pouvant être éclairci que par d'autres investigations que celles uniquement morphologiques, comme, par exemple, une analyse génétique.

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DOSE-DEPENDENT EFFECTS OF BACTERIAL ENDOTOXIN TREATMENT ON MEMBRANE PERMEABILITY OF LIVER MITOCHONDRIA IN ETHANOL-FED RATS

CORNELIU TARBA[*] and ION V. DEACIUC[**]

SUMMARY. − Male Sprague-Dawley rats were maintained for 14-15 weeks on a Lieber-DeCarli liquid diet, supplemented isocalorically with 6% ethanol. One day before the decisive experiment, a part of the animals from both the control (pair-fed) and the ethanol-fed group were injected with different concentrations of lipopolysaccharide (LPS; *E. coli* O26:B2), also known as bacterial endotoxin (0.2, 0.8, 1.5 and 3 mg/kg body weight), while their counterpart groups received only saline injections. 24 hours after the injection of LPS or saline solution the rats were anesthetised, their livers perfused with collagenase and the hepatocytes isolated by centrifugation. Mitochondria were obtained from the homogenised hepatocytes by differential centrifugations. Appropriate aliquots were suspended in 4 flasks containing a basal swelling medium and kept at $30^{O}C$ until thermal equilibration. At this moment, 5 mM succinate and different concentrations of calcium chloride (0, 10, 50 and 250 µM) were added to the 4 flasks, initiating a swelling process of mitochondrial matrix. At different times of incubation (0, 5, 10, 20 and 30 min), aliquots of 0.5 ml were extracted, placed in appropriate cuvettes and swelling monitored by the absorbance decrease recorded at 540 nm. Statistically significant differences between ethanol-fed and pair-fed rats were observed at higher Ca^{2+} concentrations (50 and 250 µM) after 30 min of incubation, the swelling being more pronounced in mitochondria of ethanol-fed rats. Differences were also seen in LPS-injected rats, both in pair-fed and in ethanol-fed animals. The extent of swelling, expressed as percent of absorbance decrease was significantly smaller, especially for the rats injected with 0.8 mg LPS/kg body weight (b.w.), suggesting a decreased membrane permeability. Differences were observed for incubation times varying from 5 to 30 min, mainly for low concentrations of calcium (0 and 10 µM). Similar but somewhat smaller differences were observed for rats injected with 0.2 mg LPS/kg b.w., whereas for rats injected with 3 mg LPS/kg b.w. there were no significant differences; however, in this last case the percent absorbance decrease (*i.e.*, swelling) tended to be larger than for the saline-injected rats. Although at least one literature account reports on the increased capacity of liver mitochondria isolated from rats injected with 0.5 mg LPS/kg body weight to maintain a high membrane potential, it is not clear yet whether this type of behaviour reflects a general decrease of membrane permeability, with physiological significance.

Chronic ethanol consumption leads in many cases to specific hepatic structural and functional alterations known under the generic name of alcoholic liver disease (ALD). How exactly the ethanol induces the disease is not entirely known. Whether ALD is determined by the nutritional defects induced by alcohol or by its direct

[*] *Department of Biology, Babeş-Bolyai University, 3400 Cluj-Napoca, Romania. Email: cntarba@biochem.ubbcluj.ro*
[**] *Department of Internal Medicine, Chandler Medical Center, University of Kentucky, Lexington, KY 40536, USA*

hepatotoxic effect is still a matter of debate. Despite strong evidence supporting the role of nutritional deficiency [10, 16, 32], it has been shown in some cases that ALD progresses towards its worst manifestation, *i.e.* liver cirrhosis, if the diet contained enough ethanol to provide 50% of the in-taken calories, even though the experimental animals were fed a balanced nutritional liquid diet [26, 27].

Excessive alcohol consumption has been known to be associated with a series of intracellular stresses, particularly detrimental to mitochondria [1-3, 8, 20, 42, 44]. One of the possible ways of alcohol attack may be through distorting the oxidant/antioxidant balance of the cell. Oxidative stress, associated with chronic ethanol consumption, leads to the depletion of mitochondrial glutathione, decreased synthesis of the respiratory chain components and acetaldehyde adduct formation [2, 3, 11, 18, 25, 45]. The result is an increase in the concentration of reactive oxygen species (ROS), such as superoxide anion, hydroxyl and peroxy-radicals, which are considered to be among the main causes of triggering a drastic change in the mitochondrial membrane permeability, known as the mitochondrial permeability transition (MPT). The formation of the so-called permeability transition pore (PTP), which represents the structural basis for the drastic change in membrane permeability, seems to be a central event in different types of cell death, either apoptosis (programmed cell death) or necrosis [14, 17, 21, 22, 24, 29, 33, 37, 38, 42, 44].

For decades, alcoholic liver disease has been attributed to necrotic events associated, among others, with the production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) [5, 30, 31, 44]. Relatively recently, however, several reports appeared, showing that ethanol, either in chronic consumption or added to isolated cells can increase appreciably the rate of hepatic apoptosis [6, 22, 33, 35]. What exactly determines the type of cell death (apoptosis or necrosis) is not perfectly understood, although it seems to be related finally to the amount of ATP produced by mitochondria, as described elsewhere [23, 36, 42]. Properties such as the rate and the intensity of the triggering agents, as well as the presence/absence of certain factors having sensitising, modulating or even protective effects determine the final outcome. The same is true in the case of ALD (see [44] for a review). Among other factors, for example, the excessive alcohol consumption increases the absorption from the gut of bacterial lipopolysaccharide (LPS), an endotoxin produced by Gram-negative bacteria, which is associated with the stimulation of the cytokine secreting cells (the hepatic macrophages or Kupffer cells). LPS seems to exert its effects mainly through TNF-related events [4, 5, 7, 15, 28], although contradictory results are sometimes reported by different authors [34, 38]. Calcium has also been reported to mediate certain metabolic effects of endotoxicosis, but the controversy is present even here (see [40] and the references therein).

As alluded above, the mitochondrion is involved in the cell death, especially in the physiological one (programmed cell death or apoptosis). This fact has been extensively documented during the last decade, the most conspicuous event in this process being the permeability transition (PT) of the inner mitochondrial membrane. This event is accompanied by matrix swelling and inability of the organelle to maintain its membrane potential. Consequently, the respiratory control and the capacity

of mitochondria for performing oxidative phosphorylation (i.e., respiration-dependent ATP synthesis) are compromised [3, 18, 21, 42]. Since ethanol was shown to increase the rate of hepatic apoptosis, as also mentioned above, it would be of interest to monitor the matrix swelling of mitochondria from ethanol-fed rats and see how well this parameter correlates with other apoptotic features.

As part of a complex study aimed at testing the effects of different pro- and anti-inflammatory cytokines/growth factors, we monitored the effect of chronic ethanol consumption on the swelling process in mitochondria from both LPS-injected and normal (saline-injected) rats and performed other biochemical/molecular biology investigations. Rather unexpected but very interesting data were obtained with regard to matrix swelling. A short report on our swelling results has already been presented [43]. A more complete presentation of the swelling data and the discussion of their possible significance make the object of the present study, whereas the rest of the results will be published elsewhere.

Material and methods. *Animals and treatment protocols.* Male Sprague-Dawley rats were kept under aseptic conditions in the animal facility of the Chandler Medical Center (University of Kentucky, Lexington) and divided into 2 large groups. The group of ethanol-fed rats was accustomed to a liquid diet (Lieber-DeCarli type) whose ethanol concentration was gradually increased from 0 to 6% during a 5-day period, after which the diet was continued for a period of 14-15 weeks. The control or pair-fed rats were kept under similar conditions but the ethanol was replaced isocalorically with maltose-dextrose. 24 hours before the decisive experiment, a part of the animals from both the pair-fed (PF) and the ethanol-fed (EF) group were injected intravenously with different concentrations (0.2, 0.8, 1.5 and 3.0 mg/kg body weight) of lipopolysaccharide (LPS), also known as bacterial endotoxin, while their counterpart (sub)groups received only sterile saline injections.

Preparation of mitochondria. One day after the injection of either LPS or saline solution the rats were anesthetised with nembutal and the livers were perfused *in situ* with collagenase as previously described [5, 39]. The resulting material was centrifuged in the cold (4°C, 2 min, 50 g), the hepatocytes resulted were suspended in fresh perfusion medium, recentrifuged and resuspended in isolation medium for mitochondria. These were obtained by differential centrifugations, essentially according to Johnson and Lardy [19]. Finally, the mitochondria were suspended in an appropriate medium, hereby called the washing and suspending medium (at a concentration of 20-30 mg protein/ml) and kept on ice until use.

Incubation of mitochondria and estimation of matrix swelling. Aliquots of 3 mg mitochondrial protein were suspended in four 25-ml Erlenmayer flasks, in a basal swelling medium, and placed in a water bath shaker at 30°C. After thermal equilibration, additions were made from concentrated stock solutions so as to have 5 mM succinate (sodium salt) in each flask and different concentrations of calcium chloride in the four flasks (0, 10, 50 and 250 μM, respectively). The final concentration of mitochondria was in all cases 1 mg/ml. At 0, 5, 10, 20 and 30 min after calcium addition, 0.5-ml aliquots were extracted from each flask, placed into an appropriate spectrophotometer cuvette and the

absorbance recorded at 540 nm. Swelling was later estimated from the recorded data as percent of the differential absorbance decrease, $(\Delta A/A_O)\cdot 100\%$, where $\Delta A = A - A_O$, A being the absorbance at a certain calcium concentration and at a given time of incubation and A_O the absorbance at 0 calcium concentration and 0 time.

Chemicals and media. The chemicals used were of analytical grade, most of them purchased from Sigma. The liquid diet was obtained from BioServ (Frenchtown, N.J.) and LPS (*Escherichia coli* O26:B6) from Difco Laboratories (Detroit, MI). The perfusion medium was Hanks bicarbonate buffer. The isolation medium for mitochondria contained 275 mM sucrose, 10 mM MOPS (pH 7.3) and 1 mM EDTA, while the washing and suspending media lacked the chelating agent. The basal swelling medium, in which mitochondria were suspended for thermal equilibration, consisted of 100 mM KCl, 40 mM sucrose, 10 mM K^+-HEPES (pH 7.3), 10 mM potassium phosphate and 10 μM rotenone.

Results. Our data, calculated as percent of differential absorbance decrease, for different (sub)groups of animals, are presented in Tables 1 to 3 along with basic statistical parameters. Table 1 compares the results obtained with mitochondria isolated from the saline-injected animals of pair-fed (PF) and ethanol-fed (EF) rats (*i.e.*, subgroups 1 and 2). Tables 2 and 3 present alternatively the results obtained for the PF (subgroups 3, 5, 7, 9) and EF rats (subgroups 4, 6, 8, 10) injected with increasing concentrations of LPS. In order to facilitate the comparison between the different subgroups, a synthesis of the results from Tables 2 and 3 are presented in Table 4, in terms of differences between means, along with the corresponding coefficient of statistical confidence (p).

The presentation of percent differential absorbance decrease instead of absolute absorbance data was preferred because of the large individual variations within the same subgroup. Since the source of variability resides in the first place in different starting values of absorbance (*i.e.*, different absorbance values at time 0, especially as the concentration of added calcium increases), a good way to diminish the impact of inter-individual (and inter-run) variability is to compare not absolute absorbance values and not even absolute absorbance differences but to calculate and compare **differential** absorbance decreases, $\Delta A/A_O$, where $\Delta A = A - A_O$, A being the absorbance at a certain calcium concentration and at a given time of incubation and A_O the absorbance at 0 calcium concentration and 0 time. Rather large calcium-dependent differences at 0 time may arise from small errors in reading times, due to the exponential decay of the absorbance curve (*i.e.*, the decay is much faster immediately after the addition of calcium and slows gradually).

As can be seen from Table 1, there is a tendency towards larger absorbance decreases (differences) in mitochondria obtained from ethanol-fed rats as compared to the pair-fed animals, although the differences are statistically significant only after 30 min of incubation at 50 μM calcium or 20-30 min at 250 μM calcium (in general, a smaller final absolute absorbance value means a larger absorbance decrease, hence a higher degree of swelling). To our knowledge, there is only one account in the literature dealing directly with this type of study in ethanol-fed rats (Pastorino *et al.* [38]) and it has reported a significant increase in the mitochondrial swelling induced by moderate concentrations of calcium (and other agents), more obvious than in our case.

On the other hand, if comparisons are made between LPS-injected and saline-injected animals, for either PF or EF rats, there is an obvious decrease in the extent of mitochondrial swelling when lower LPS concentrations are used (see Tables 2 and 4) and an increase in swelling at higher concentrations of LPS (see Tables 3 and 4). Such observations have not yet been mentioned in the literature, although there is one study performed by Guidot [13] which reports on the existence of an increased respiratory capacity and a higher membrane potential in mitochondria of ethanol-fed rats injected with 0.5 mg LPS/kg body weight. These observations can be easily related in terms of the chemiosmotic theory of energy coupling to a smaller extent of matrix swelling (smaller membrane permeability) and, consequently, to a higher degree of coupling.

If the mean values of the percent differential absorbance decreases for certain selected subgroup pairs at different calcium concentrations are plotted as a function of incubation time, as presented in Figs. 1 to 5, the differences between subgroups become more obvious.

For clarity, in Fig.1, the results at one concentration of calcium in each of the saline-injected subgroups (pair-fed and ethanol-fed, respectively) have been omitted. There is a clear divergence between the two sets of curves, indicating a larger degree of swelling in the ethanol (EtOH)-fed group, although, as we know from Table 1, the differences are statistically significant only for longer incubation times (20-30 min) and larger calcium concentrations (50 and 250 μM).

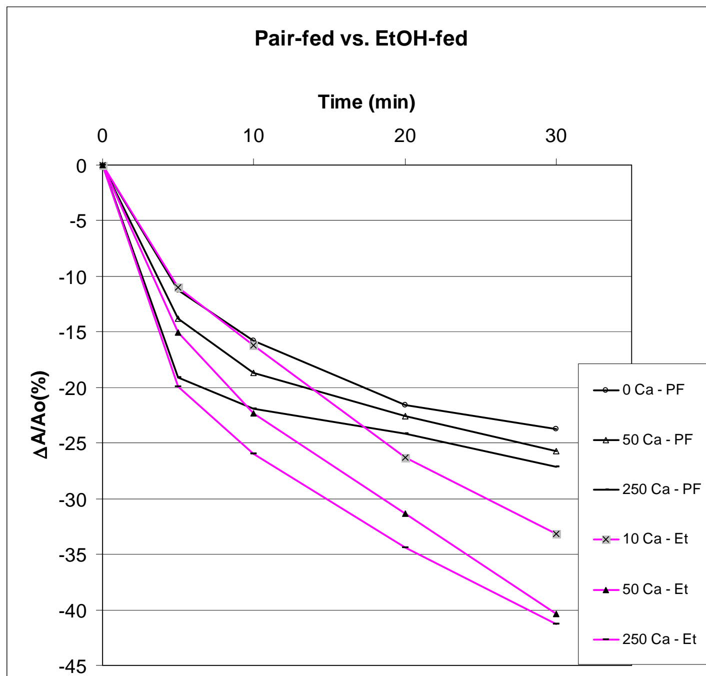


Fig. 1. *Comparison of mitochondrial swelling in ethanol (EtOH)-fed (Et) and pair-fed (PF) groups.*

Table 1

Absorbance decreases recorded in the presence of variable [Ca^{2+}] with mitochondria of ethanol-fed rats as compared to pair-fed rats

The data were calculated as differential percent decrease [$(\Delta A/A_0)\cdot 100\%$] as described in the experimental section. The meaning of the symbols appearing in the first column is as follows: PF = pair-fed; Sal = saline-injected; EF = ethanol-fed; SEM = standard error of mean.

Type of treatment (Group)		0 μM Ca^{2+}				10 μM Ca^{2+}				50 μM Ca^{2+}				250 μM Ca^{2+}			
		5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min
1 = PF + Sal		Differential absorbance decrease (%)															
Rat #	2	- 1.83	- 5.09	- 7.24	- 10.98	- 3.50	- 9.86	- 10.18	- 12.89	- 5.01	- 7.64	- 10.26	- 15.27	- 7.80	- 9.31	- 10.82	- 15.59
	4	- 7.30	- 10.04	- 13.87	- 16.11	- 9.90	- 12.43	- 16.18	- 17.92	- 8.67	- 11.71	- 15.53	- 18.14	- 15.53	- 17.85	- 21.75	- 23.99
	5	- 7.36	- 13.18	- 20.48	- 21.25	- 11.78	- 17.39	- 19.28	- 24.26	- 14.03	- 18.93	- 23.35	- 25.32	- 21.18	- 22.16	- 23.77	- 27.07
	51	- 20.76	- 26.12	- 34.28	- 36.23	- 20.83	- 27.10	- 34.53	- 37.15	- 21.49	- 28.56	- 33.55	- 36.29	- 26.18	- 30.57	- 33.55	- 35.38
	52	-19.21	- 24.38	- 32.16	- 34.18	- 19.54	- 25.29	- 31.11	- 33.79	- 20.06	- 26.67	- 30.39	- 33.46	-24.71	- 29.87	- 30.85	- 33.66
$\bar{X}_{PF}$		- 11.29	- 15.76	- 21.61	- 23.75	- 13.11	- 18.41	- 22.26	- 25.20	- 13.85	- 18.70	- 22.62	- 25.70	- 19.08	- 21.95	- 24.15	- 27.14
± SEM		3.70	4.09	5.19	4.96	3.20	3.41	4.59	4.59	3.18	4.07	4.38	4.11	3.36	3.96	3.98	3.56
2 = EF + Sal																	
Rat #	7	- 15.72	- 21.60	- 34.16	- 42.42	- 14.87	- 20.99	- 34.41	- 39.44	-13.53	- 19.30	- 32.40	- 39.14	- 14.38	- 18.87	- 29.85	- 38.41
	7'	- 9.72	- 19.44	- 29.44	- 38.89	- 7.50	- 15.07	- 28.40	-39.17	- 9.17	- 18.68	- 29.72	- 39.44	- 12.64	- 22.57	- 31.87	- 40.83
	9-12	- 4.94	- 6.66	- 9.13	- 11.87	- 10.64	- 12.63	- 16.20	-20.86	- 22.44	- 29.03	-32.26	- 42.62	- 32.74	- 36.51	- 41.59	- 44.47
		# 11				# 10				# 9				# 12			
$\bar{X}_{EF}$		- 10.13	- 15.90	- 24.24	- 31.06	- 11.00	- 16.23	- 26.34	- 33.16	- 15.05	- 22.34	- 31.46	- 40.40	- 19.92	- 25.98	- 34.44	- 41.24
± SEM		3.18	4.66	7.68	9.65	2.14	2.48	5.36	6.15	3.90	3.35	0.87	1.11	6.43	5.37	3.62	1.76
$\bar{X}_{EF} - \bar{X}_{PF}$		1.16	- 0.14	- 2.63	- 7.31	2.11	2.18	- 4.08	- 7.96	- 1.20	- 3.64	- 8.84	- 14.70	- 0.84	- 4.03	- 10.29	- 14.10
p		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	< 0.05	ns	ns	< 0.05	< 0.05

Table 2

Absorbance decrease recorded with mitochondria obtained from PF or EF rats injected with 0.2 and 0.8 mg LPS/kg body weight

Type of treatment (Group)		0 µM Ca^{2+}				10 µM Ca^{2+}				50 µM Ca^{2+}				250 µM Ca^{2+}			
		5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min
3		Differential absorbance decrease (%): PF + LPS (0.2 mg/kg body weight)															
Rat #	13	- 1.21	- 2.11	- 2.17	- 4.89	- 3.86	- 4.65	- 5.25	- 5.76	- 4.65	- 5.91	- 9.41	- 12.91	- 34.04	- 39.35	- 41.58	- 42.67
	14	- 1.96	- 3.14	- 4.98	- 7.24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	- 9.02	- 10.14	- 12.04	- 14.77
	15	- 2.28	- 3.56	- 4.67	- 6.60	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	- 8.53	- 9.35	- 11.04	- 12.50
	16	- 1.05	- 2.04	- 0.64	- 2.79	- 8.09	- 8. 84	- 9.08	- 11.52	- 16,06	-25.83	- 32.17	- 33.92	- 36.65	- 39.38	- 42.87	- 45.08
	53	- 6.83	- 10.14	- 16.51	- 18.63	- 6.75	- 10.28	- 16.59	- 18.62	- 8.48	- 11.79	- 18.02	- 19.52	- 13.14	- 16.14	- 20.12	- 21.55
$\bar{x}_3$		- 2.67	- 4.20	- 5.80	- 8.03	- 6.23	- 7.92	- 10.31	- 11.97	- 9.73	- 14.51	- 19.86	- 22.12	- 20.27	- 22.87	- 25.53	-27.31
± SEM		1.07	1.51	2.80	2.76	1.25	1.69	3.33	3.72	3.35	5.91	6.63	6.20	6.22	6.83	7.00	6.93
4		Differential absorbance decrease (%): EF + LPS (0.2 mg/kg body weight)															
Rat #	17	- 1.48	- 2.19	- 2.41	- 2.91	- 3.67	- 4.06	- 4.61	- 4.88	- 1.59	- 3.84	- 6.03	- 6.31	- 33.50	- 39.64	- 42.43	- 44.30
	20	- 0.75	- 1.45	- 2.42	- 3.00	- 2.09	- 2.84	- 3.38	- 3.65	- 1.66	- 2.63	- 4.29	- 6.87	- 25.55	- 29.47	- 32.69	- 33.12
	49	- 3.09	- 7.78	- 26.08	- 37.87	- 3.95	- 9.84	- 32.66	- 38.67	- 12.01	- 34.43	- 40.33	- 48.51	- 36.84	- 43.25	- 47.59	- 49.54
$\bar{x}_4$		- 1.77	- 3.81	- 10.30	- 14.59	- 3.24	- 5.58	- 13.55	- 15.73	- 5.09	- 13.63	- 16.88	- 20.56	- 31.96	- 37.45	- 40.90	- 42.32
± SEM		0.69	2.00	7.89	11.64	0.58	2.16	9.56	11.47	3.46	10.40	11.73	13.97	3.35	4.13	4.37	4.84
5		Differential absorbance decrease (%): PF + LPS (0.8 mg/kg body weight)															
Rat #	21	- 1.00	- 1.21	- 1.36	- 2.77	- 0.87	- 0.87	- 0.92	- 2.14	- 8.35	-15.24	- 19.77	- 22.03	-29.12	- 32.34	-35.81	-39.34
	22	- 0.68	- 1.30	- 2.25	- 2.99	- 1.01	- 1.58	- 3.15	- 5.24	- 11.10	- 24.34	- 26.25	- 28.62	- 33.24	- 39.21	- 41.41	- 45.13
	24	- 0.46	- 1.08	- 2.11	- 2.91	- 1.43	- 1.54	- 2.75	- 3.26	- 3 .59	- 4.85	- 8.61	- 14.42	- 30.96	- 35.92	- 40.59	- 42.25
	50	- 6.10	- 9.23	- 14.58	- 16.94	- 6.63	- 9.68	- 14.64	- 17.01	- 7.10	- 10.15	- 14.43	- 16.95	- 11.60	- 13.66	- 16.79	- 19.39
$\bar{x}_5$		- 2.06	- 3.21	- 5.07	- 6.40	- 2.49	- 3.42	- 5.37	- 6.91	- 7.53	- 13.64	- 17.27	- 20.51	- 26.23	- 30.28	- 33.65	- 36.53
± SEM		1.36	2.01	3.17	3.51	1.39	2.09	3.13	3.43	1.56	4.15	3.76	3.13	4.95	5.72	5.75	5.83
6		Differential absorbance decrease (%): EF + LPS (0.8 mg/kg body weight)															
Rat #	25	- 2.20	- 2.91	- 3.62	- 4.16	- 4.10	- 4.39	- 5.23	- 5.76	- 4.63	- 10.39	- 14.07	- 27.32	-37.77	- 42.22	- 46.38	- 49.70
	28	+ 1.02	+ 0.57	- 0.17	- 0.51	+ 0.85	+ 0.34	- 0.40	- 1.14	+ 0.17	- 0.91	- 3.07	- 6.25	- 23.02	- 27.12	- 31.95	- 34.11
$\bar{x}_6$		- 0.59	- 1.17	- 1.88	- 2.33	- 1.63	- 2.03	- 2.79	- 3.45	- 2.23	- 5.65	- 8.57	16.78	- 30.40	- 34.67	- 39.17	- 41.91
± SEM		1.61	1.74	1.73	1.83	2.47	2.37	2.42	2.31	2.40	4.74	5.50	10.53	7.38	7.55	7.22	7.79

Table 3

Absorbance decrease recorded with mitochondria obtained from PF or EF rats injected with 1.5 and 3.0 mg LPS/kg body weight

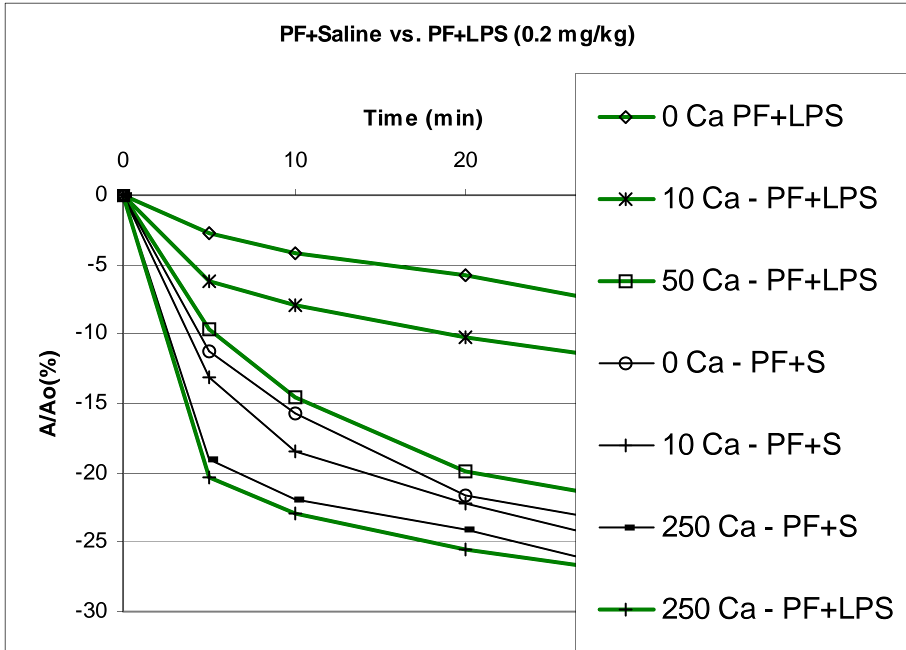
Type of treatment (Group)		0 µM Ca^{2+}				10 µM Ca^{2+}				50 µM Ca^{2+}				250 µM Ca^{2+}			
		5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min
7		Differential absorbance decrease (%): PF + LPS (1.5 mg/kg body weight)															
Rat #	31	+ 1.21	+ 0.66	+ 0.36	- 0.24	- 0.60	- 0.90	- 1.56	- 2.04	- 1.20	- 3.12	- 11.99	- 20.08	- 24.76	- 32.01	- 36.93	-38.43
	32	- 5.65	- 13.50	- 17.08	- 19.42	- 5.79	- 11.50	- 17.97	- 20.94	- 15.15	- 17.56	- 20.94	- 22.79	- 24.24	- 28.17	- 30.92	- 32.71
	47	- 16.58	- 20.51	- 26.45	- 28.47	-16.53	- 20.15	- 27.11	- 29.73	- 19.38	- 25.56	- 30.26	- 33.77	- 22.71	- 26.22	- 29.13	- 32.10
$\bar{x}_7$		- 7.00	- 11.11	- 14.39	- 16.47	- 7.64	- 10.85	- 15.55	- 17.57	- 11.91	- 15.41	- 21.06	- 25.55	-23.90	- 28.80	- 32.33	- 34.41
± SEM		5.18	6.23	7.85	7.92	4.69	5.57	7.47	8.17	5.49	6.56	5.27	4.18	0.62	1.70	2.36	2.02
8		Differential absorbance decrease (%): EF + LPS (1.5 mg/kg body weight)															
Rat #	35	- 3.22	- 10.38	- 26.34	- 30.65	- 3.55	- 11.26	- 26.06	- 30.11	- 10.93	- 25.41	- 32.35	- 35.57	- 29.67	- 33.77	- 38.42	- 40.60
	36	- 22.61	- 26.13	- 34.81	- 38.19	- 27.58	- 33.02	- 39.92	- 45.23	- 24.07	- 30.10	- 37.40	- 39.52	- 32.23	- 33.09	- 41.51	- 44.50
	45	- 15.74	- 19.19	- 25.04	- 27.27	- 16.03	- 20.19	- 26.16	- 28.26	- 16.21	- 21.12	- 26.39	- 28.44	- 22.12	- 26.45	- 29.90	- 32.65
$\bar{x}_8$		- 13.85	- 18.56	- 28.73	- 32.04	- 15.72	- 21.49	- 30.71	- 34.53	-17.07	- 25.54	- 32.05	- 34.51	- 28.01	- 31.10	- 36.61	- 39.25
± SEM		5.67	4.56	3.06	3.23	6.94	6.32	4.60	5.37	3.82	2.59	3.18	3.25	3.04	2.33	3.47	3.49
9		Differential absorbance decrease (%): PF + LPS (3 mg/kg body weight)															
Rat #	39	- 15.08	- 20.89	- 27.78	- 29.62	- 15.15	- 20.95	- 28.19	- 30.58	-17.27	- 24.09	- 28.40	- 30.44	-22.19	- 27.44	- 30.99	- 32.35
	40	- 15.32	- 24.11	- 29.22	- 31.84	- 15.67	- 23.69	- 29.29	- 32.62	- 15.75	- 24.33	- 27.30	- 31.70	- 21.35	- 28.08	- 29.57	- 33.33
$\bar{x}_9$		- 15.20	- 22.50	- 28.50	- 30.73	- 15.41	- 22.32	- 28.74	- 31.60	- 16.51	- 24.21	- 27.85	- 31.07	- 21.77	- 27.76	- 30.28	- 32.84
± SEM		0.12	1.61	0.72	1.11	0.26	1.37	0.55	1.02	0.76	0.12	0.55	0.63	0.42	0.32	0.71	0.49
10		Differential absorbance decrease (%): EF + LPS (3 mg/kg body weight)															
Rat #	41	- 15.68	- 24.03	- 30.34	- 34.66	- 23.36	-29.71	- 33.47	- 37.84	-25.85	- 30.91	- 37.27	- 41.19	- 32.38	- 36.65	- 41.70	- 44.21
	43	- 9.90	- 18.35	- 22.90	- 25.18	- 14.34	-18.95	- 24.01	- 25.18	- 17.13	- 20.95	- 25.57	- 27.51	- 21.40	- 25.01	- 27.52	- 30.13
$\bar{x}_{10}$		- 12.79	- 21.19	- 26.62	- 29.92	- 18.85	-24.33	- 28.74	- 31.51	- 21.49	- 25.93	- 31.42	- 34.35	- 26.89	- 30.83	- 34.61	- 37.17
± SEM		2.89	2.84	3.72	4.74	4.51	5.38	4.73	6.33	4.36	4.98	5.85	6.84	5.49	5.82	7.09	7.04

Table 4

Differences between mean values and their statistical significance

Mean values from Tables 2 and 3 are compared by calculating the differences between selected groups and the corresponding statistical confidence coefficient (p)

Differences between group means	0 μM Ca^{2+}				10 μM Ca^{2+}				50 μM Ca^{2+}				250 μM Ca^{2+}			
	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min	5 min	10 min	20 min	30 min
$\bar{x}_3 - \bar{x}_1$	8.62	11.56	15.81	15.72	6.88	10.39	11.95	13.23	4.12	4.19	2.76	3.58	- 1.19	- 0.92	- 1.38	- 0.17
p	≥ 0.05	< 0.05	< 0.05	< 0.05	ns	< 0.05	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_5 - \bar{x}_1$	9.23	12.55	16.54	17.35	10.62	14.99	16.89	18.29	6.32	5.06	5.35	5.19	- 7.15	- 8.33	- 9.50	- 9.39
p	ns	< 0.05	< 0.05	< 0.05	< 0.05	< 0.02	< 0.05	< 0.05	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_7 - \bar{x}_1$	4.29	4.65	7.22	7.29	5.47	7.46	6.71	7.63	1.94	3.29	1.56	0.15	- 4.82	- 6.85	- 8.18	-7.27
p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_9 - \bar{x}_1$	- 3.91	- 6.74	- 6.89	- 6.98	- 2.30	- 4.01	- 6.48	- 6.40	- 2.66	- 5.51	- 5.23	- 5.52	- 2.69	- 5.81	- 6.13	- 5.70
p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_4 - \bar{x}_2$	8.36	12.09	13.94	16.47	7.76	10.65	12.79	17.43	9.96	8.71	14.58	19.89	- 12.04	- 11.47	- 6.46	- 1.08
p	≥ 0.05	ns	ns	ns	< 0.05	< 0.05	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_6 - \bar{x}_2$	9.54	14.73	22.36	28.73	9.37	14.20	23.55	29.71	12.82	16.69	22.89	23.62	- 10.48	- 8.69	- 4.73	- 0.67
p	ns	ns	ns	ns	ns	< 0.05	< 0.05	< 0.05	ns	≥ 0.05	< 0.02	ns	ns	ns	ns	ns
$\bar{x}_8 - \bar{x}_2$	- 3.72	- 2.66	- 4.49	- 0.98	- 4.72	- 5.26	- 4.37	- 1.37	- 2.02	- 3.20	- 0.59	5.89	- 8.09	- 5.12	- 2.17	- 1.99
p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_{10} - \bar{x}_2$	- 2.66	- 5.29	- 2.38	1.14	- 7.85	- 8.10	- 2.40	1.65	- 6.44	- 3.59	0.04	6.05	- 6.97	- 4.85	- 0.17	4.07
p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_4 - \bar{x}_3$	0.90	0.39	- 4.50	- 6.56	2.99	2.34	- 3.24	-3.76	4.64	0.88	2.98	1.56	-11.69	- 14.58	- 15.37	- 15.01
p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
$\bar{x}_5 - \bar{x}_2$	8.07	12.69	19.17	24.66	8.51	12.81	20.97	26.25	7.52	8.70	14.19	19.89	- 6.31	- 4.30	0.79	4.71
p	< 0.05	< 0.05	< 0.05	< 0.05	< 0.02	< 0.02	< 0.02	< 0.02	ns	ns	< 0.02	< 0.01	ns	ns	ns	ns



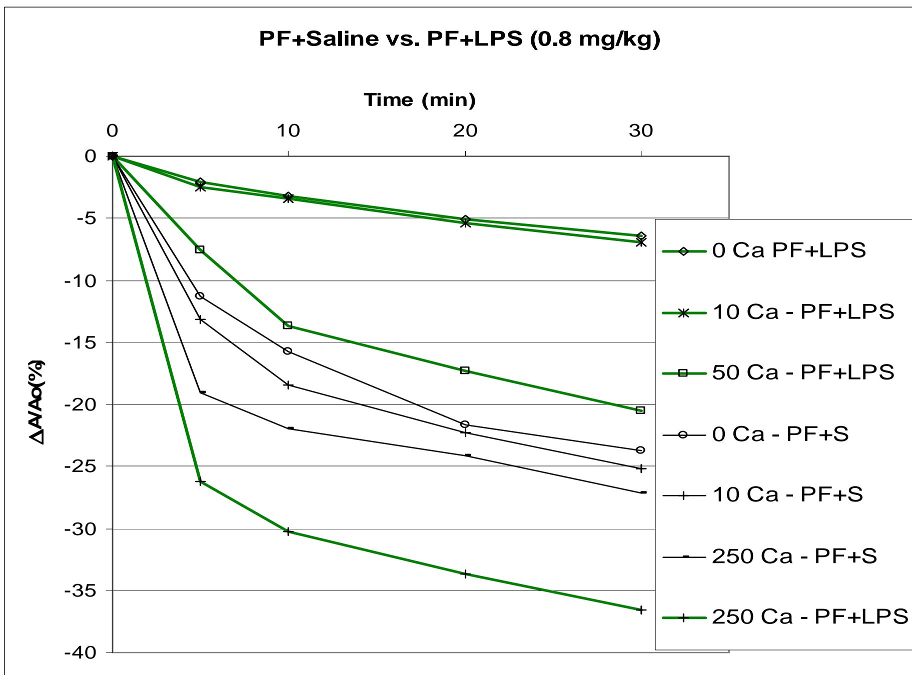
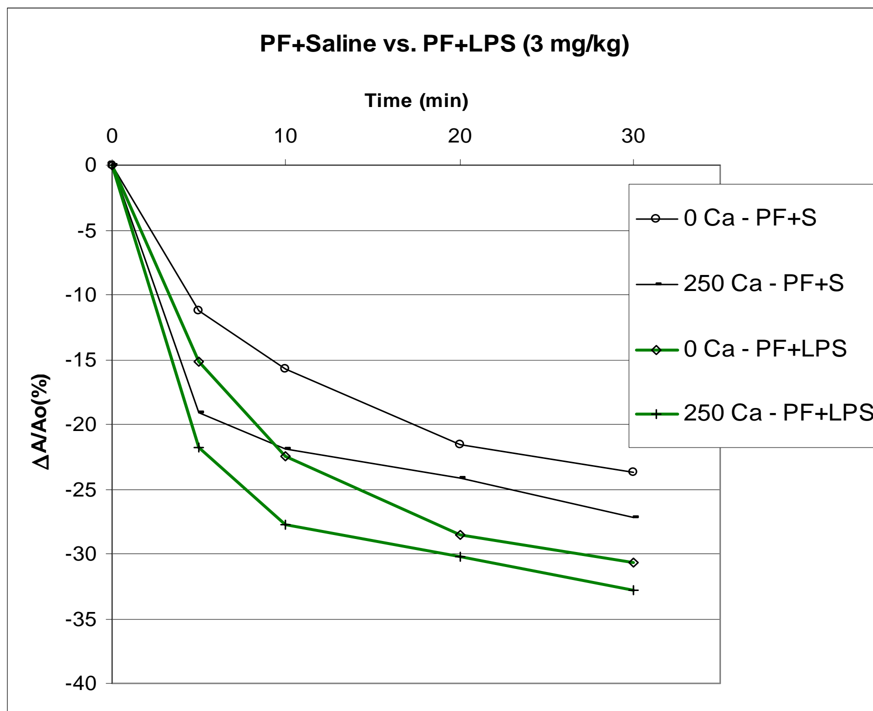


Fig. 2 (up) and Fig. 3 (down). *Effect of rat treatment with small doses of LPS on mitochondrial swelling.*

The results are presented as percent of differential absorbance decrease at different times. Calcium concentrations (μM) and the type of subgroup are as detailed in the attached window.

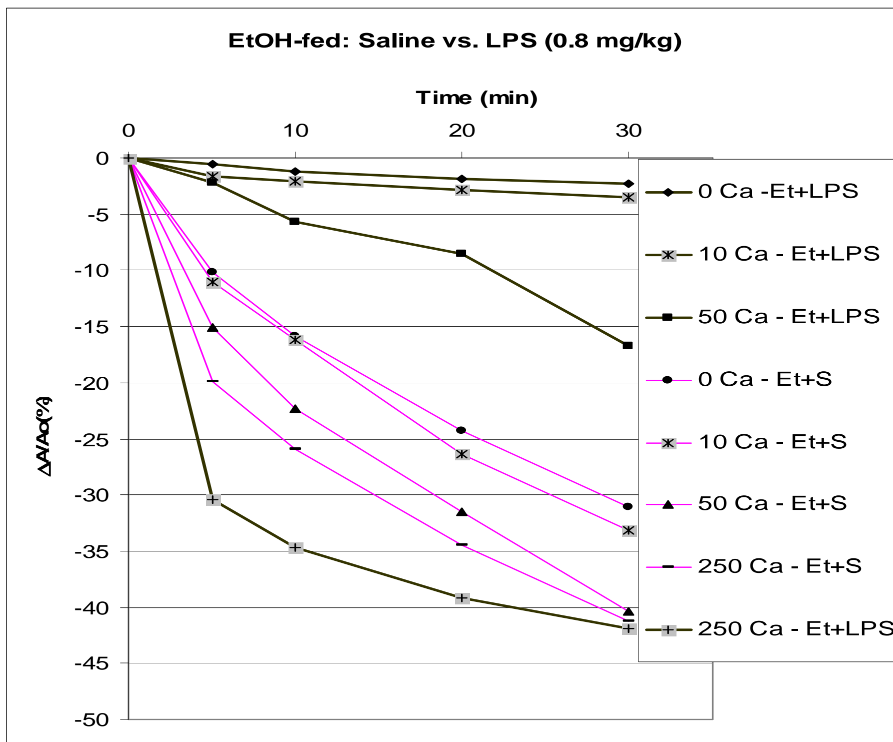
The pre-treatment of rats with small doses of LPS (0.2 and 0.8 mg/kg) determines a very interesting swelling behaviour pattern of mitochondria, as shown in Figs.2 and 3, above. For clarity, the curves obtained with 50 μM calcium concentration for saline-injected rats have been omitted in both figures. As can be seen from the figures, the mitochondria of LPS-injected rats in the presence of small concentrations of calcium (close to the physiological ones) are resistant to swelling, the differential absorbance decrease after 30 min of incubation being somewhere in the interval of 5 to 10%, while for mitochondria from the corresponding saline-injected rats the absorbance decrease is close to 25%. The differences between the LPS- and saline-injected rats are in fact statistically significant ($p < 0.05$) beginning with 10 min of incubation. Even at 50 μM calcium, the degree of swelling is smaller for mitochondria of LPS-injected rats, although the differences are not statistically significant in this case. At the same time, one can see that at the highest calcium concentration used (250 μM), which is far from the physiological range of the cytoplasmic calcium, the extent of swelling is larger for the LPS-treated rats, although still not statistically significant. This trend becomes more and more obvious as LPS concentration used is increased, as can be seen from Fig.4.



F i g . 4 . *Effects of rat treatment with a high dose of LPS (3 mg/kg b.w.) on mitochondrial swelling.*

Again, for clarity reasons, only two curves from each group of mitochondria were shown in Fig.4. Even though the differences are not statistically significant, based on the general trend, we consider that the differences are real. The situation for 1.5 mg LPS/kg body weight, which is intermediate, has not been graphically presented because of the large degree of overlapping between the two sets of curves.

If swelling behaviour of mitochondria obtained from ethanol-fed rats is compared with respect to the effect of LPS pre-treatment, one can observe a similar pattern of response as with pair-fed animals, although certain differences are also present. The similarity is due to the effect of LPS, whereas the differences are due to the effect of ethanol. An illustrative example is given in Fig.5, which presents the absorbance changes in mitochondria of ethanol-fed, saline-injected rats vs. LPS-injected rats (0.8 mg/kg body weight).



F i g . 5 . *Swelling of mitochondria obtained from ethanol-fed rats: saline-injected (S) vs. LPS-injected animals (0.8 mg/kg b.w.)*

Because of the steeper absorbance decrease in mitochondria obtained from ethanol-fed, saline-injected rats (see also Fig.1), the differences between the two groups of rats, at least for small doses of LPS (such as in Fig.5), at low and medium calcium concentrations, is even more evident than in the case of pair-fed rats. The converse is true, however, for the highest calcium concentration (cf.

Figs.2 and 3) and for the higher doses of LPS (1.5 and 3 mg/kg; not presented here), in which cases the absorbance decrease is much steeper at the beginning and levels off towards the end of the monitoring period (20-30 min), as can be deduced from Tables 3 and 4. As expected, even more impressive differences can be seen in Table 4 if one compares subgroups 5 and 2, *i.e.* the PF rats injected with 0.8 mg LPS/kg b.w. and the EF saline-injected rats.

Discussion. The mitochondrial permeability transition (MPT) mentioned above as a crucial point in a cell's life and death decision is followed by matrix swelling, a phenomenon that can be monitored by measuring the absorbance change (decrease) at 540 nm (or at a nearby wavelength). Indeed, our measurements on mitochondria obtained from the liver of chronically ethanol-fed rats show a higher propensity of these organelles to undergo swelling, as compared to those of the pair-fed group, although not so evident as in the few other studies on this problem existing in the literature [37, 39]. However, there are factors which may explain the differences, such as a different composition of the swelling medium and a different design of the experiment. In any case, a moderate increase in membrane permeability and the associated moderate swelling could be compatible with an apoptotic death, which is a controlled process requiring energy [23, 36], whereas an extensive swelling should lead to membrane breaking and loss of any capacity for ATP synthesis and for further control of the dying process. The consequence should be death by necrosis.

The real surprise in our study came when we looked at the results obtained with mitochondria of LPS-treated rats. There is a clear pattern that emerges from the study of the differential absorbance decrease recorded in hepatic mitochondria from rats given different doses of LPS. The most conspicuous effect is that obtained with mitochondria from rats treated with 0.8 mg LPS/kg body weight. The absorbance decrease in this case is much smaller than in mitochondria of saline-injected rats. On the other hand, at the highest dose tested (3 mg LPS/kg b.w.), the situation is reversed, even though the increase in absorbance does not appear as statistically significant. The dose of 0.2 mg LPS/kg has similar effects to that of 0.8 mg, although slightly less evident, while 1.5 mg LPS induces a higher change (this time an increase), but less than that observed with 3 mg LPS. Such an extensive study on LPS dose dependency has not yet been performed, but our results do agree, indirectly, with the only study existing in the literature in this respect. Guidot [13] has studied the effect of two doses of LPS (0.5 mg and 2 mg LPS/kg) on mitochondrial respiratory capacity and membrane potential in rat hepatocytes isolated from endotoxin-pretreated rats. The decreased swelling (our results at 0.2-0.8 mg LPS) and the increased membrane potential (at 0.5 mg LPS, in Guidot's study) reflect a decreased permeability of mitochondrial membrane, whereas an increased swelling (our results at 3 mg LPS) and the decreased membrane potential (2 mg LPS in [13]) reflect an increased membrane permeability. How could these effects be explained? It is known that a variety of oxidative injuries, such as those induced by hyperoxia

and sublethal doses of LPS, induces resistance to a subsequent and otherwise lethal oxidative stress [9, 41]. Also, in a related study [12], Guidot determined that endotoxin treatment in rats in a dose-time dependent fashion, that is known to induce tolerance to hyperoxia-mediated lung injury, increased nonenzymatic scavenging of superoxide anion by lung mitochondria. Thus, our results could be explained, at least in part, by the antioxidant effect induced by the sublethal doses of LPS, which translates into a protective effect of membrane phospholipids, which are known to be very sensitive to oxidative stress [25].

One additional observation is that, at least for rats given endotoxin 24 hrs prior to sacrifice, protective doses should be considered only up to approximately 1 mg/kg body weight. Doses above these values or acting for a longer period of time probably increase the oxidative stress, initiate the permeability transition and increase the matrix swelling. The consequence is the uncoupling of oxidative phosphorylation, the decreased capacity for ATP synthesis and, finally, death, either by apoptosis or by necrosis, depending on the extent of the effects.

Conclusions. Chronic ethanol feeding of rats induces a moderate increase in the matrix swelling, a phenomenon which is compatible with (and which could lead to) an increased rate of apoptosis. The treatment with LPS of both pair-fed and ethanol fed rats has two different results, depending on the dose: a decreased permeability (decreased swelling) at low doses and an increased permeability (increased swelling) at moderate doses. The first result is a clear indication of the protective (most likely, antioxidant) effect of sublethal doses of endotoxin, whereas the second should be related to the apoptotic/necrotic effects induced by higher doses of endotoxin.

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THE GROWTH AND SEED PRODUCTION OF SOYBEAN PLANTS CULTIVATED ON MINE SPOILS

SILVIA ONAC*

SUMMARY. – The growth and seed production of two soybean (*Glycine max*) cultivars, Agat and Diamant, cultivated in mine spoils from Cavnic (Baia Mare mining area, Romania) were studied, under field conditions. Three experimental variants were organised: V_1 – control (unpolluted soil), V_2 – 50% spoils+50% unpolluted soil, V_3 – 100% spoils. No fertilizers have been added to any of the cultivation substrata. Seed germination was not affected by the heavy metals in spoils. The growth of both soybean cultivar plants cultivated in 100% spoils, as well as the root elongation, were strongly inhibited, and the amount of dry matter in the leaves increased. The plants cultivated in 50% spoils had the best growth. In all three cultivation variants the Agat cv. plants had a better growth in the first part of the vegetation cycle, later being overtopped by the Diamant cv. plants. For both soybean cultivars grown in 100% spoils, the number of pods and seeds was very low. The plants grown in 50% spoils had a high number of pods and seeds, but an increased percentage of undeveloped seeds.

A number of heavy metals (like Zn, Cu, Fe) are essential for plant growth and development, but when present in excess are strongly phytotoxic. Others, however, such as cadmium and lead, are toxic even in small concentrations and are not known to have any functional value in plants. Heavy metals induce many biochemical and physiological alterations in plant cells and visibly inhibit the plant development in general. Stunted growth, leaf epinasty, and chlorosis are striking symptoms of strong metal toxicity [21].

Mine spoils from Cavnic (a Pb-Zn-Cu mine from Baia Mare mining area, Romania) contain a high amount of heavy metals [13]. The purpose of this study was to evaluate the growth and seed production of two soybean cultivars, Agat and Diamant, cultivated in these spoils and whether these processes could be improved by mixing the spoils with soil.

Materials and methods. A field experiment was conducted using two soybean [*Glycine max* (L.) Merrill] cultivars, Agat and Diamant, created by the Agricultural Research Station, Turda, Romania. The experiment was described in a previous paper [13] and lasted one vegetation cycle. The seed germination capacity was determined in the laboratory. 200 soybean seeds for each variant and cultivar were used, distributed in four germinators, with 50 seeds each. The germination substrata were the three experimental variants (control, 50% spoils+50% unpolluted soil, 100% spoils).

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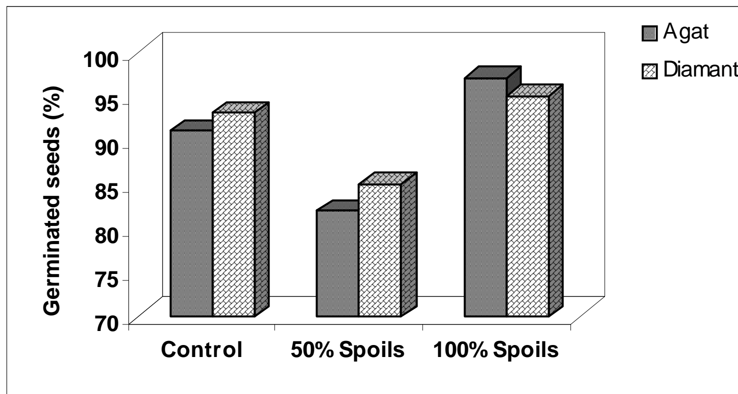
*Babeş-Bolyai University, Department of Plant Physiology, 3400 Cluj, Romania.
E-mail: sonac@bioge.ubbcluj.ro

The growth of soybean plants was estimated by measuring the length of stems and roots and by determining the accumulation of dry matter. The stem length was periodically measured, at 26, 45, 60, 77, 94 and 108 days. For measuring the root length, 12-day-old plants, grown in the laboratory, were used. The accumulation of dry matter in the leaves of soybean plants was estimated in different vegetation stages, namely the beginning of flowering, S1, the beginning of pod genesis, S2, and the stage of fruit ripening, S3. Disc samples 1.3 cm diameter wide were sectioned from the sampled leaves, being first weighed when fresh, then oven-dried at 105°C, and weighed again. The results were related to 100 g fresh matter.

In order to estimate the fruit and seed production, the total number of fruits and seeds from 15 soybean plants within each cultivar and experimental variant was taken into consideration.

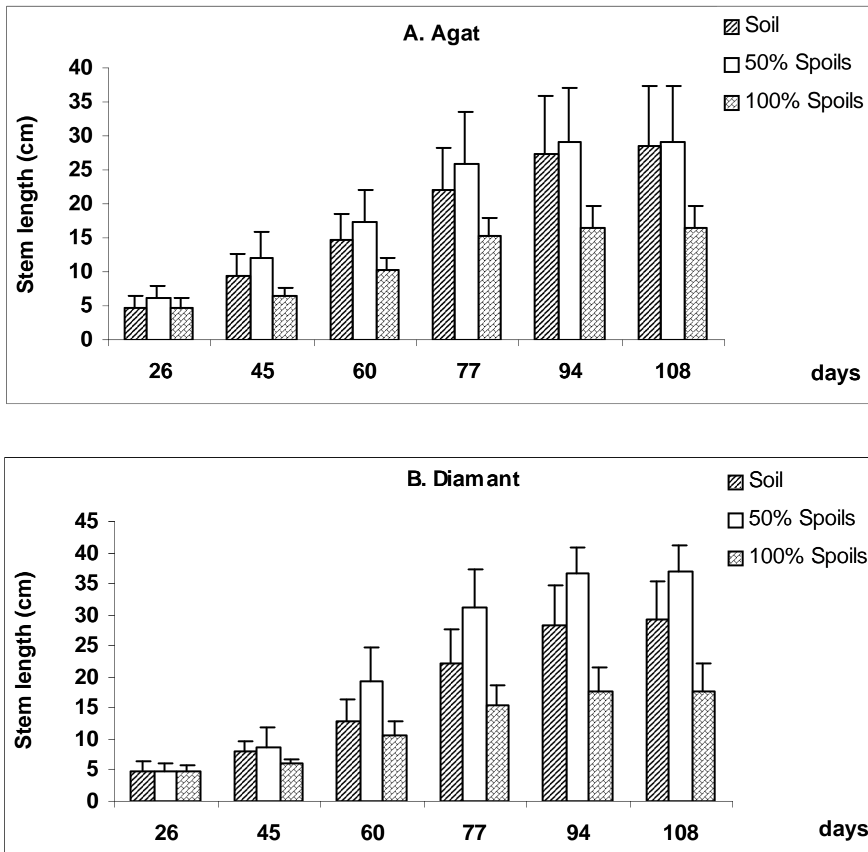
Results and discussion. The seeds of both soybean cultivar plants germinated in all the experimental variants, as follows. The Agat cv. seeds germinated as 91% in the control, 82% in 50% spoils, and 97% in 100% spoils. The Diamant cv. seeds germinated as 93% in the control, 85% in 50% spoils, and 95% in 100% spoils (Fig. 1).



F i g. 1. *Variation of the germination capacity of soybean seeds, Agat and Diamant cvs., depending on the germination substratum.*

For both soybean cultivars, a high percentage of germinated seeds in the 100% spoil variant was found, slightly higher than the control, which might suggest that heavy metals from spoils do not negatively affect seed germination [3], but might stimulate it [10]. The sandy consistency of spoils could explain this high percentage. For both cultivars, the lowest percentage of germinated seeds was found in 50% spoils. The seeds of Diamant cv. from the control and 50% spoil groups germinated in greater number than those of Agat cv., whereas in 100% spoils the situation was inverted. However, the differences between the two soybean cultivars were not large in any of the experimental variants.

Despite the high germination rate, the growth of both soybean cultivar plants cultivated in 100% spoils was strongly inhibited. The growth of plants in 50% spoils was stimulated, particularly in the case of Diamant cv. In the first two weeks after sowing, the plants cultivated in 100% spoils, both cultivars, had the best growth. At the age of 26 days the plants of both cultivars and all variants had approximately the same height and starting with the age of 45 days the plants from 100% spoils had a much slower growth when compared to the control and especially to plants grown in 50% spoils. In the case of Diamant cv., plant growth was reduced to half when compared to plants grown in 50% spoils. In both cultivars the plants from 100% spoils grew intensely between the ages of 45 and 77 days, after which their growth slowed dramatically. The control plants and those from 50% spoils grew constantly to the age of 94 days (Fig. 2).

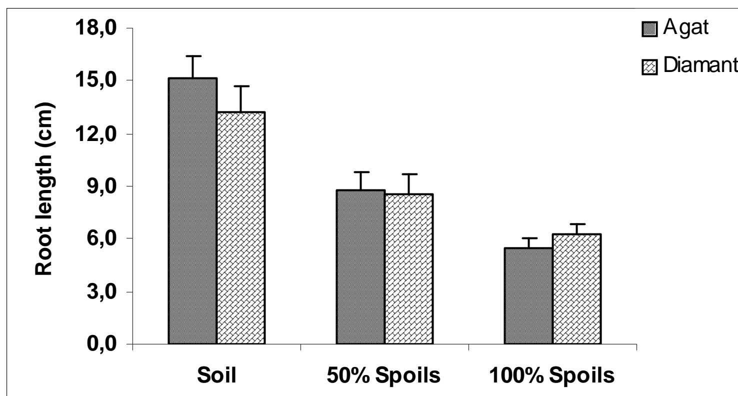


F i g. 2. *The growth of soybean plants, Agat and Diamant cvs., cultivated in soil, a mixture of soil (50%) and spoils (50%), and spoils (100%), for 108 days (n=15).*

In all three cultivation variants the Agat cv. had a better growth in the first 50 days after sowing, but later the growth of the Diamant cv. was better, particularly in 50% spoils. This overtopping by Diamant cv. plants suggests a greater sensitivity of the Agat cv. plants, regardless the cultivation substratum. It should be noted that no fertilizers have been added to any of the cultivation substrata.

The first evident effect of metal toxicity in plants is considered to be the reduction of root elongation. A r d u i n i *et al.* [1] found that Cd, especially in combination with Cu, strongly inhibited the root growth of *Pinus pinea* and *Pinus pinaster* seedlings, affecting root morphology (colour and hair development) and architecture (the pattern of branching, the lateral root number, the length). Heavy metals inhibit the root elongation and the growth and development of plants, thus decreasing their biomass production [6, 9, 12, 14, 15, 22].

Our results regarding the influence of heavy metals on the growth of soybean plant roots are in accordance with the literature data (Fig. 3). The elongation of the plant roots cultivated in 100% spoils was greatly inhibited in the case of both Agat and Diamant cvs. (64 and 52%, respectively) when compared to the control. Root inhibition was noticed in plants cultivated in 50% spoils, but in a smaller percentage when compared to the control (42 and 35%, respectively).As with the growth of shoots, Agat cv. seems more sensitive regarding root elongation, at least in the case of the variants with spoils.



F i g. 3. *The root elongation of soybean plants, Agat and Diamant cvs., cultivated in soil, a mixture of soil (50%) and spoils (50%), and spoils (100%), for 12 days (n=15).*

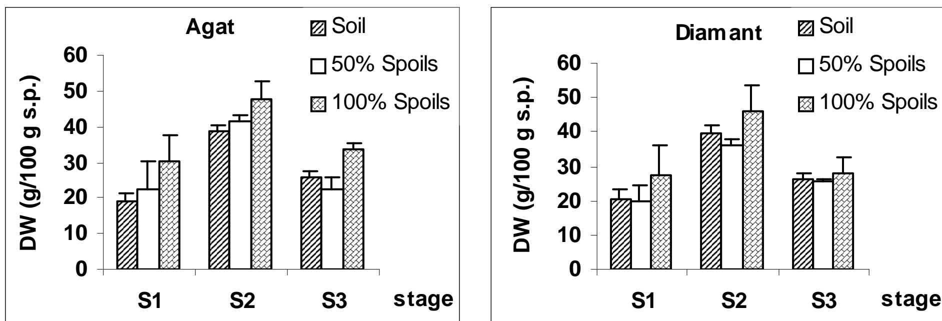
Studying the plants growing on ore bodies enriched in Cu, L a n a-r a s *et al.* [11] found that the high Cu concentration in the ore soil in combination with the low Ca level resulted in a strong inhibition of the growth of the plants. P ä t s i k k ä *et al.* [16] observed that excess Cu applied *in vivo* caused the decrease of both root and shoot growth of bean plants. Long-term exposure to high levels of Zn reduced the growth of bean plants, as a consequence of inhibition of both photosynthesis and translocation of photosynthetic products [17]. Cd inhibits plant growth, probably

because of its impact on uptake of nutrients and water, therefore affecting metabolic processes [14]. O u z o u n i d o u *et al.* [15] found a significant reduction in Ca and Fe content in the tissues of maize treated with excess Cu.

Certain changes in root morphology, such as inhibited elongation and enhanced lateral root formation [8], might be related to the strong decrease in indolyl-3-acetic acid oxidase activity in roots exposed to high heavy metal concentrations [12]. The reduction of root growth is attributed to the inhibitory effect of heavy metals on cell extension, possibly caused by the changes in cell wall characteristics, and to the lowering of mitotic activity [22]. Heavy metals reduce Ca uptake by plants, which is required for cell extension and division, by replacing it at its binding sites on the exterior surface of the plasma membrane [9, 12].

The stunted growth of plants cultivated on highly polluted soils may be due to a specific toxicity of the metals, antagonism with other nutrients, or inhibition of root penetration in the soil. If root elongation is restricted, then nutrients such as P, K, Fe, etc. could fall to growth-limiting levels [8].

The accumulation of dry matter in the leaves of soybean plants, Agat and Diamant cvs., is showed in Fig. 4. In all three vegetation stages and for both studied soybean cultivars, an increase in the amount of dry matter in the leaves of plants cultivated in 100% spoils was observed.

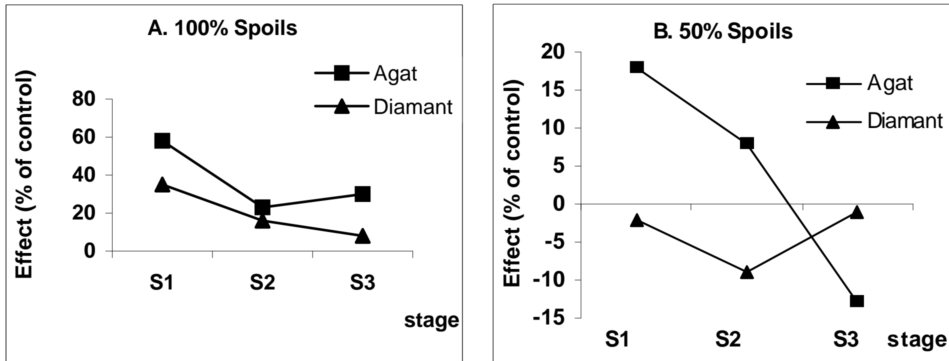


F i g. 4. *Dry matter (dry weight, DW) accumulation in the leaves of soybean plants, Agat and Diamant cvs.,in three vegetation stages: S1= the beginning of flowering, S2= the beginning of pod genesis, S3= fruit ripening (n=3).*

For the Agat cv. plants this amount was 58, 23, and 30 % higher in S1, S2 and S3, respectively, compared to the control. For the Diamant cv. plants the amount of dry matter in leaves was 35, 16, and 8% higher, respectively (Fig. 5, A). The highest difference between the control and the plants cultivated in 100% spoils in regards to the accumulation of dry matter in leaves was found in S1 for both soybean cultivars.

The soybean plants, both cultivars, grown in 50% spoils reacted differently regarding the accumulation of dry matter. For the Agat cv. plants the amount of dry matter increased with 18 and 8% in S1 and S2, respectively, compared to the control. In S3, however, it decreased by 13% compared to the control (Fig. 5, B). The increase of dry matter in S1 and S2 was much lower in comparison to plants

cultivated in 100% spoils. For the Diamant cv. the dry matter content of the plants grown in 50% spoils decreased slightly with 2, 9, and 1% in S1, S2, and S3, respectively, compared to the control.

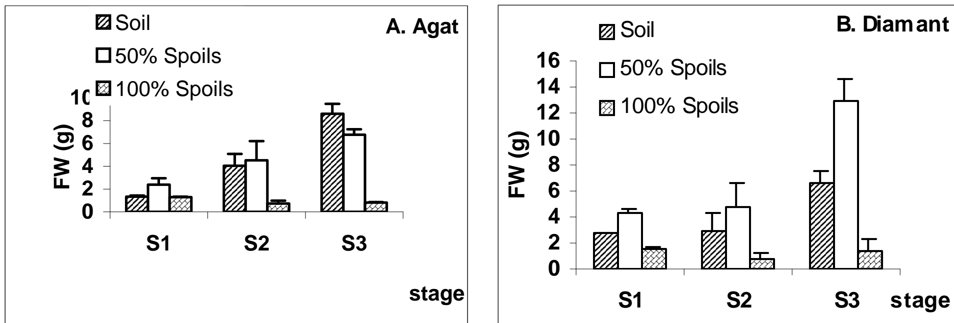


F i g. 5. *The influence of heavy metals from 100% spoils (A) and 50% spoils (B) on the accumulation of dry matter (% of the control) in the leaves of soybean plants, Agat and Diamant cvs.,in three vegetation stages: S1= the beginning of flowering, S2= the beginning of pod genesis, S3= fruit ripening.*

The percentage of dry matter usually increases with the age of the plant due to the accumulation of storage compounds and of a relative increase in the proportion of structural material (cell walls and lignin) [12]. The highest content of dry matter in soybean leaves, in all the cultivation variants and both cultivars, occurred at about 85 days after sprouting. Later a decrease was recorded, as a consequence of the translocation of reserve compounds towards seeds. The young leaves of plants use an important amount of the photosynthetic compounds for their growth. As they reach the maturity stage, their needs decrease. As a result, leaves become important in producing the energy needed for the reduction of nitrates, the synthesis of macromolecules, the renewal of cytoplasmic proteins, and for the nutrient transport. Consequently, most of the assimilates are translocated from leaves into fruits and seeds [5].

For both soybean cultivars studied in our experiment the increase of dry matter amount in the leaves of plants cultivated in 100% spoils did not indicate a real increase of the biomass. The amount of fresh matter of these plants was very low (Fig. 6).

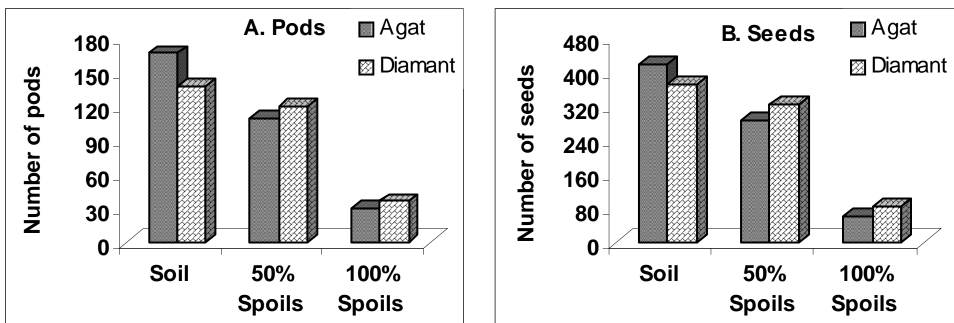
This effect is probably due to a heavy metal-induced disturbance in the water balance of the plants, leading to water stress [3, 4, 10, 20], to the uptake and translocation of heavy metals, as well as to the increased accumulation of proteins (phytochelatins). Water stress caused by many heavy metals leads to all the other abnormalities in physiological and metabolic processes [19]. Cadmium affects the uptake and distribution of nutrients in plants and inhibits the water transport to shoots, producing a water deficiency in plants [7, 9, 18]. By inhibiting cell extension and division, heavy metals may cause a reduction of cell water content, leading to an increase in dry weight/fresh weight ratio (DW/FW) [9].



F i g. 6. *Fresh matter (fresh weight, FW) accumulation in the leaves of soybean plants, Agat and Diamant cvs.,in three vegetation stages: S1= the beginning of flowering, S2= the beginning of pod genesis, S3= fruit ripening (n=15).*

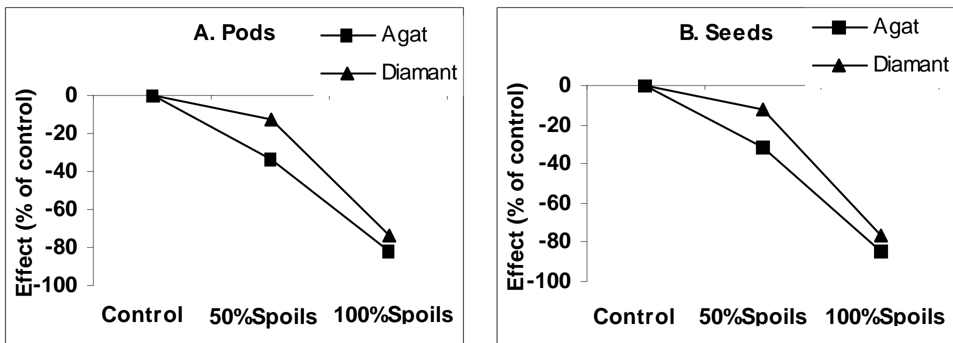
G r e g e r [9] found that when the concentration of nutrients decreased in relation to Cd concentration, the DW/FW ratio increased. The study concluded that the growth of sugar-beet plants was affected by the proportion of Cd to nutrient concentration and not directly by the Cd content in the plants. This is in agreement with our results, which showed that the Diamant cv. plants had a better growth when compared to the Agat cv. plants, though their leaves and roots contained higher amounts of heavy metals [13]. The mine spoils used in our experiment as cultivation substratum for soybean plants were nutrient-deficient [13]. This nutrient deficiency could explain the stunted growth of soybean plants [2]. By mixing with soil, the quality of spoils is improved by dilution of heavy metals and enrichment of nutrients. Consequently, the DW/FW in the 50% spoil variant was lower as compared to the 100% spoil variant, and the growth of soybean plants was much better.

The soybean plants of both cultivars cultivated in 100% spoils fructified, but their number of pods and seeds was much lower when compared to the control (Fig. 7).



F i g. 7. *The total number of pods (A) and seeds (B) of 15 soybean plants/experimental variant/cultivar.*

The control plants of Diamant cv. had less pods and seeds than those of Agat cv., but more pods in spoil variants. The difference between the two cultivars was, however, not great in any of the cultivation variants. The difference between the number of pods and seeds of Agat cv. plants from spoil variants and that of the control was higher when compared to Diamant cv. plants. The number of pods of Diamant cv. plants decreased 13 and 74% compared to the control in the 50% spoil variant and 100% spoil variant, respectively. The number of pods of Agat cv. plants decreased 34 and 82%, respectively, compared to the control. Similarly, the number of seeds of Diamant cv. plants decreased 13 and 77% compared to the control in the 50% and 100% spoil variants, respectively. The decreasing of seed number of Agat cv. plants was 32 and 85%, respectively, compared to the control (Fig. 8).

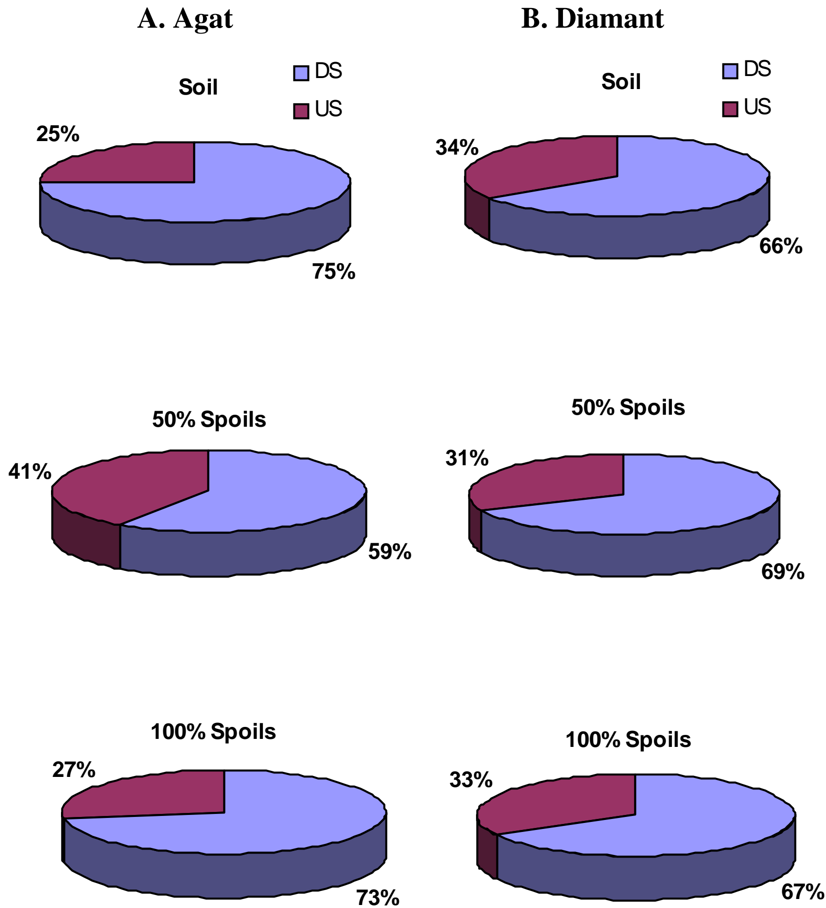


F i g. 8. *The influence of heavy metals from 50% spoils and 100% spoils on pod (A) and seed (B) production of soybean plants, Agat and Diamant cvs.*

The plants of both soybean cultivars had a percentage of undeveloped seeds, smaller than the percentage of developed seeds (Fig. 9). The highest percentage (41%) of undeveloped seeds was recorded for the Agat cv. plants cultivated in 50% spoils. The control plants of Diamant cv. and those cultivated in 100% spoils had a higher percentage of undeveloped seeds (34 and 33%, respectively) than the plants of Agat cv. cultivated in the corresponding variants (25 and 27%, respectively).

Although the soybean cultivars Agat and Diamant grown in 100% spoils reached the maturity and fructified, the number of pods and seeds was low when compared to the control. There was an average of 2 pods/plant for both cultivars, compared to 11 pods/plant for Agat cv. control and 9 pods/plant for Diamant cv. control. There was an average of 4 seeds/plant, Agat cv., and 5 seeds/plant, Diamant cv., compared to 28 and 25 seeds/plant, respectively, for control.

Our results suggest not only the reduction of the toxicity of heavy metals from spoils by mixing the spoils with soil, but also a stimulation of growth. This was observed in the soybean plants cultivated in 50% spoils. These plants had a better growth even when compared to the control. In addition, the number of pods and seeds exhibited little decrease, though the percentage of undeveloped seeds increased.



F i g. 9. *The percentage of developed (DS) and undeveloped seeds (US) from the pods of soybean plants, Agat and Diamant cvs., grown in various experimental variants.*

Conclusions. 1. For both studied soybean cultivars, Agat and Diamant, the percentage of germinated seeds in 100% spoils was high. This may suggest that heavy metals from spoils do not negatively affect seed germination. The sandy consistency of spoils could explain this high percentage. The lowest percentage of germinated seeds was found in 50% spoils, for both soybean cultivars.

2. The growth of both soybean cultivar plants cultivated in 100% spoils was strongly inhibited. The growth of plants in 50% spoils was stimulated, particularly in the case of Diamant cv. In all the experimental variants the Diamant cv. plants had a better growth than those of Agat cv.

3. The elongation of the plant roots cultivated in 100% spoils was much inhibited, in the case of both Agat and Diamant cvs., whereas in 50% spoils this inhibition was less marked. The inhibition of root elongation is higher for Agat cv. plants in both spoil variants.

4. For both soybean cultivars studied in our experiment the dry matter amount in the leaves of plants cultivated in 100% spoils increased. In the 50% spoil variant this increase is diminished. The increase of dry matter amount did not indicate a real increase of the biomass.

5. The soybean plants, Agat and Diamant cvs., cultivated on 100% spoils reached maturity and fructified, but the number of pods and seeds was very low when compared to the control. The plants of Diamant cv. cultivated on spoil variants had a greater number of pods and seeds than the plants of Agat cv. from the corresponding variants.

6. The effect of heavy metals and/or nutrient deficiency from Cavnic spoils on the growth and seed production of soybean plants was reduced by mixing the spoils with soil in equal percentages.

7. The Diamant cv. plants seemed to be more resistant to the harmful cultivation substratum represented by mine spoils, when compared to the Agat cv. plants.

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BIOCHEMICAL ACTIVITY OF SOME URBAN SOILS IN EASTERN SIBERIA

ELIZAVETA V. NAPRASNIKOVA[*] and VALERIAN A. SNYTKO*

SUMMARY. - Biochimical activity, assessed as urease activity, was determined in soils of three East Siberian towns located in the Prebaikalia: Irkutsk, Shelekhov and Sayansk. Unpolluted soils in the Olkhon Island of the Baikal Lake seved for comparison. Ranges ofthe pH values for optimum urease activity were found to be 6.3-7.4 (in Olkhon Island soils), 7.3-7.9 (in Irkutsk soils), 7.5-8.3 (in Shelehkov soils) and 7.0-7.8 (in Sayansk soils). At the same pH or at nearly the same pH, soil urease activity presented the order: olkhon Island > Irkutsk > Shelekov > Ssayansk.

For stuying biological activity in urban soils, enzymological methods were also applied. Thus, enzyme activities were measured in soils of Warsaw (Poland) [16], Trier and Bonn-Bad Godesberg (Germany) [15], Oulu (Finland) [10], Dőrsten (Germany) [3], Moscow (Russia) [11], Serpukhov (Russia) [12], Brno and Podolí (Czech Republic) [13], Salzburg (Austria) [14], Kiel, Rostock, Eckernfőrde, Halle/Saale and Stuttgart (Germany) [4], Rostov-Don (Russia) [2], Lvov (Lemberg) (Ukraine) [5] and in soils of the Siberian towns of Sharipova [6,8], Irkutsk and Shelekov [9] and Sayansk [7].

In the present paper we describe the investigations carried out for comparison of biochemical activity, assessed as urease activity, in soils of three towns (Irkutsk, Shelekhov and Sayansk) located in Eastern Siberia, in the Prebaikalia and in unpolluted soils in the Olkhon Island of the Baikal Lake.

Site description. The town of Irkutsk is the biggest industrial centre in Prebaikalia. It was founded some 300 years ago on forest soils. The forests surrounding this town are dominated by larch and birch.

Shelekhov is a new town at 8 km from Irkutsk. This town is located on an area of pine-larch forests and is affected by pollutants (mainly fluoride) from an aluminium smalter.

The town of Sayansk and the neighbouring areas occupy a part of the Irkutsk-Cheremkhovo plain. The soils on this plain are soddy-podzolic and gray forest soils. The chemical factory "Sayanskkhimprom" emits many pollutants (polyvinyl chloride dust, sulphur dioxide, nitrogen dioxide etc.)

The Olkhon Island in the Baikal Lake is considered unpolluted. The landscapes here are natural, almost not disturbed by local economic activity. The soils are steppe and forest soils.

[*] *Siberian Branch of the Russian Academy of Sciences, Institute of Geography, Ulanbatorskaya Street 1, Irkutsk 664033, Russia. E-mail: root@irigs.irk.ru*

Material and methods. In each of the four areas studied (the three towns and the island), soil was sampled from the 1-11-cm depth of many 25-m^2 plots during the vegetation period.

Soil pH was measured potantiometrically. Biochemical activity of soils was assessed by determination of urease activity according to the method described in [1]. Briefly, the soil sample amended with urea is incubated. During incubation, the soil urease catalysis of urea. The time (hours) necessary for increasing air pH (estimated with universal indicator paper) by 1.5-2.0 units due to the evolved ammonia is recorded. This time is inversely proportionate to the urease activity.

The analytical data were subnitted to statistical evaluation.

Results. Fig.1 shows that the soil pH range for optimum urease activity is different in the four areas. This pH range was 6.3-7.4 in the Olkhon Island soils, 7.3-7.9 in the Irkutsk soils, 7.5-8.3 in the Shelekhov soils and 7.0-7.8 in the Sayansk soils. Statistical evaluation of the results indicated positive correlation between urease activity and pH of soils.

Fig.1 also shows that at the same pH or at nearly the same pH urease activity presented the order: Olkhon Island > Irkutsk > Shelekhov > Sayansk. It is evident from this order that the soils in Irkutsk, the oldest industrial town in the Prebaikalia, are less polluted than the soils of the newer towns of Shelekhov and Sayansk.

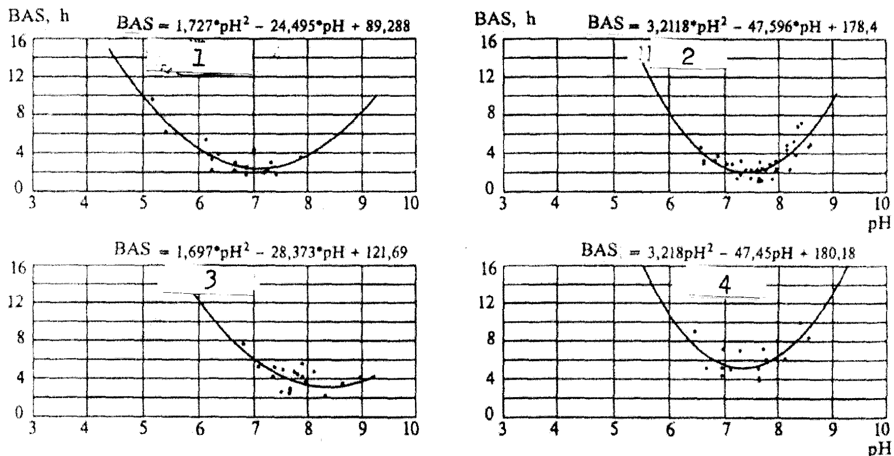

Fig. 1. *Relationship between biochemical (urease) activity and pH of soils in unpolluted and urban areas.*
Biochemical activity of soils (BAS) was assessed as urease activity and expressed in hours (h) necessary for increasing air pH by 1.5-2.0 units.
1 - Olkhon Island. 2. Irkutsk. 3 - Shelekhov. 4. Sayansk.

Conclusions. 1. The range of optimum pHs of biochemical (urease) activity was different in soils of the three urban and the unpolluted areas studied.

2. Urease activity presented the order: unpolluted Olkhon Island soils > Irkutsk soils > Shelekhov soils > Sayansk soils.

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THE PRESENCE OF SULPHATE-REDUCING BACTERIA IN THE BOTTOM SEDIMENTS OF THE ROMANIAN BLACK SEA AREA

ELENA STOICA*, MIHAIL DRĂGAN-BULARDA and JAN KUEVER*****

SUMMARY. – Seven strains (Et_1, But_1, But_2, But_3, Lac_1, $Isob_1$, $Benz_1$) of Gram-negative, mesophilic, nonsporing sulphate-reducing bacteria were isolated from bottom sediments of the Romanian Black Sea coast. Analysis of partial 16S rDNA sequences, obtained from pure cultures of isolated strains by PCR, revealed that all strains belonged to the δ- subclass of *Proteobacteria.* Three strains (But_1, But_3, Lac_1) were morphologically and nutritionally similar. According to their 16S rDNA sequences, the isolates were affiliated with the following species: *Desulfofrigus fragile* (But_1, But_3, Lac_1, 97.9–98% similarity), *Desulfovibrio acrylicus* (Et_1, 98% similarity), *Desulfobacterium autotrophicum* (But_2, 99% similarity), *Desulfobacterium niacini* ($Isob_1$, 99% similarity). Strain $Benz_1$ represents a new species of the genus *Desulfobacula* and had 96% sequence similarity to the previously described species. This is a first description of the diversity (community structure) of sulphate-reducing bacteria from the marine sediments of the Romanian Black Sea coast by molecular techniques.

The composition of bacterial communities of estuarines and coastal regions is largely unknown, despite the substantial roles many coastal bacteria play in biogeochemical cycles and the potential utility of such bacteria for bioremediation and other biotechnological applications. The lack of knowledge of this important group of microorganisms can be attributed directly to their low cultivability by standard microbiological techniques and to a reluctance on the part of marine microbiologists to study the small, potentially unrepresentative group of bacteria that can be readily cultured from coastal areas [16].

The increasingly routine use of culture-independent PCR-based methods (16S rDNA sequence comparison) in recent research has led to isolation of novel microorganisms (previously unsequenced and possibly uncultured). These methods have been applied for identification of the group of sulphate-reducing bacteria (SRB) [31].

Although several of marine bacteria have been isolated during the last years, little or nothing is known about their functional and ecological roles. Most recent reports on bacterial communities are concerned primarily with the number of

* *National Institute for Marine Research and Development ″Grigore Antipa″, Bd. Mamaia 300, RO-8700, Constanţa, Romania. E-mail: estoica@alpha.rmri.ro*

***Babeş-Bolyai University, Department of Plant Biology, 3400 Cluj-Napoca, Romania. E-mail: dragan@bioge.ubbcj.ro*

****University of Bremen, Center for Environmental Resarch and Technology, Institute for Soil Science, Leobener Strasse, D-28359 Bremen, Germany. E-mail: jkuever@uni-bremen.de*

species potentially present in different habitats, and only a few reports concern the actual abundance of specific bacteria or their impact on the environment [5, 12, 13].

Increasing eutrophication of marine coastal areas generates a higher primary production in the photic zone. The organic matter produced is accumulated mainly in the bottom sediments, which provide anaerobic conditions as a result of the active consumption of oxygen by heterotrophic organisms [22]. Under such environmental conditions the process of sulphate reduction plays a key part in the mineralisation of organic matter. According to J o r g e n s e n [18], over 50% of the accumulated organic matter becomes mineralised in coastal and shelf sediments.

Mesophilic marine sulphate-reducing bacteria (SRB), which form a phylogenetically distinct group within the *delta subclass* of *Proteobacteria*, make up an ecologically and morphologically heterogeneous group of microorganisms [4].

The main property of those either obligate or facultative anaerobic bacterial populations is their active use of sulphate as a final electron acceptor during anaerobic respiration. The final product of this respiration is hydrogen sulphide, which is discharged into the environment. Where concentrations of H_2S are very high, this can penetrate SRB cell membranes and thus impedes their metabolic activity [22].

SRB utilise a very wide spectrum of different low molecular organic compounds (lactate, acetate, propionate, succinate, pyruvate, ethanol, aliphatic acids, sugars, amino acids, indole, nicotinic acid) as electron donors, and also as carbon and energy sources. In general, the most versatile isolates from various marine habitats were originally classified as members of the genera *Desulfobacterium, Desulfobacula, Desulfococcus*, *Desulfonema*, *Desulfosarcina, Desulfospira* and *Desulfotignum* [2, 3, 6, 15, 20, 24, 27, 29-33]. Members of the genus *Desulfobacula* are characteristically restricted to the utilisation of short chain fatty acids and simple organic compounds as electron donors, whereas members of the genus *Desulfobacterium* can also grow chemoautotrophically on H_2 and CO_2 [2, 3, 6, 20, 24, 27].

SRB, which generate large amounts of toxic hydrogen sulphide in aquatic ecosystems, are important not only for ecological reasons. They are also vital from the point of view of the economy. This primarily concerns the petroleum industries, which use immense amounts of seawater in their technologies while recovering oil from under the sea bed. A large amount of SRB may cause the oil

and gas to acidify, the piping to corrode and technical installations to become clogged [4]. Owing to their quite considerable ecological and economic importance, SRB have become recently a popular subject of scientific investigations [22].

Although it is well known that sulphatereduction is a dominant process for carbon mineralisation in the Black Sea, no isolates of sulphate-reducing bacteria obtained from this habitat have been described. Despite the obviously important position of SRB in the functioning of marine ecosystems, data concerning them in coastal areas of the Black Sea are completely lacking. Therefore, the aim of the

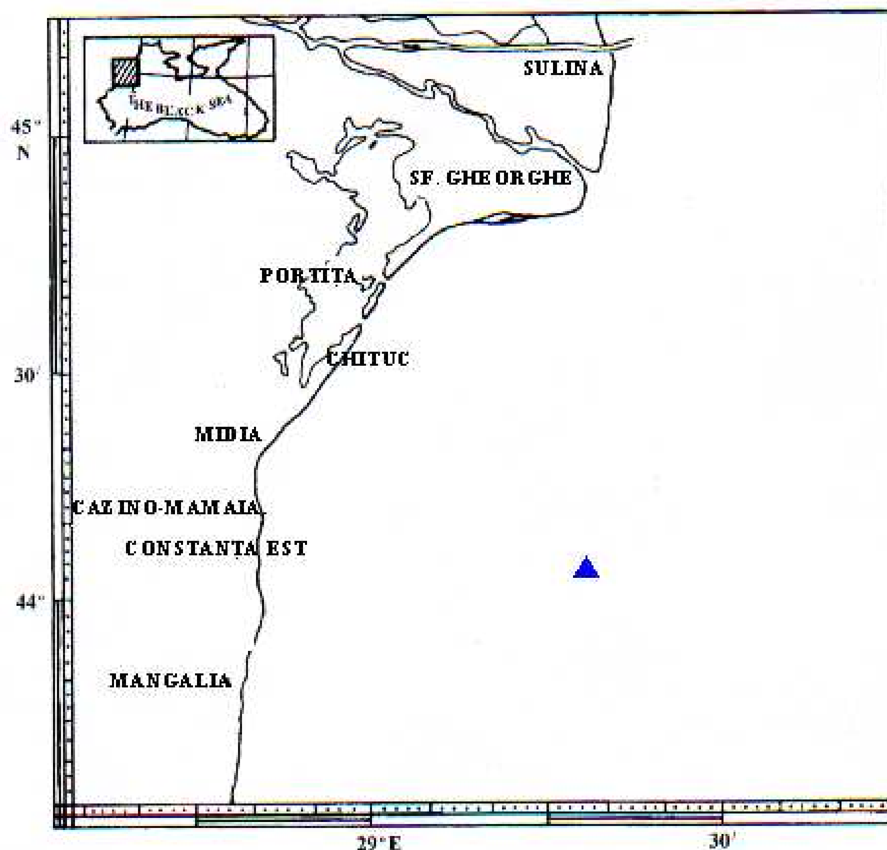
present study was the description of SRB diversity in the marine sediments of the Romanian Black Sea coast by molecular techniques. Taken into account the limited data available on the abundance of the above-mentioned SRB genera in marine sediments [21, 26], we conducted in addition a MPN (most probable number) approach with different substrates for estimating the number of different populations of marine sulphate-reducing bacteria.

Materials and methods. *Sampling site.* The investigations were carried out in the southern part of the Romanian Black Sea coast, in spring 2001. The sampling station was located at a distance to shore of 30 marine miles (East Constanßa site, $44^0$10' N/$29^0$22' E) (Fig. 1). This coastal area is strongly affected by inflow of riverine waters (Danube) and by wastewater discharges, which carry large amounts of inorganic nutrients and organic matter into the sea. During the last three decades, the inputs of inorganic nutrients and organic matter have led to the increase of the frequency and amplitude of algal blooms and to the accumulation of organic matter in the bottom sediments, followed by producing of hypoxia or, occasionally, of anoxia. Between 1970 and 1974 (warm season) the mean values of dissolved oxygen, recorded at the East Constanßa area, have decreased for the entire water layer [7, 8]. From 1975 (characteristically for the period of May-September), it was observed the decline of dissolved oxygen (frequently less than 3.0 cm^3/l equivalent to 50% saturation) in the entire 0-50 m layer, particularly below the thermocline. This reduction in dissolved oxygen has resulted in the mass mortalities of benthic communities. In good agreement with algal bloom frequency and intensity reduction, suboxic areas in the Romanian shelf were restricted after 1990 [8-11].

Source of organisms. The strains were isolated in pure culture from enrichment cultures inoculated with anaerobic sediment collected from offshore site (East Constanßa) at a water depth of 50 m.

Culture methods and media. Enrichment cultures were obtained by inoculating medium with 1 cm^3 sediment. A mineral basal medium prepared as described by W i d d e l and B a k [31] was used for enrichment, isolation and routine culture work. As electron acceptor 28 mM of sterile sodium sulphate, equivalent to the concentration in seawater, was added from a 1 M stock solution. Different substrates were added as the organic carbon/energy sources: acetate (10 mM), lactate (10 mM), butyrate (10 mM), isobutyrate (5 mM), ethanol (10 mM), propionate (10 mM), benzoate (2 mM), formate (10 mM), hexadecanoate (1 mM). $FeSO_4$ (0.2 mM) was used as an indicator of sulphate reduction: a black FeS precipitation indicated sulphide formation. The cultures were incubated at 28°C in the dark for about 3 weeks.

Viable counts of sulphate-reducing bacteria were determined by most probable number (MPN) counts [1]. For MPN enumeration, the sediment sample was used as inoculum and the substrates for the different MPN enumeration were the compounds specified above.



F i g . 1 . *Location of the sampling site (East Constanßa station).*

Isolation of bacteria. Pure cultures were obtained by repeated deep agar dilution series [31]. To check purity, the isolates were inoculated into media with 0.1% yeast extract plus H_2 , formate, lactate, pyruvate or sugars as substrates. After incubation the cultures were examined microscopically.

Substrate utilisation test. The ability to oxidise and grow on different organic compounds was tested by using the medium with the compounds (as electron donors) as described above. To test the capability of autotrophic growth, cultures were grown under a headspace of 80% H_2-20% CO_2 at an overpressure of 101.3 kPa. Samples containing no electron donor served as controls.

PCR amplification and sequencing of the 16S rRNA gene. To amplify the almost complete 16S rRNA encoding gene (1,500 bp) of strains, primers GM3F and GM4R were used in a 35-cycle PCR with an annealing temperature of 40 °C [23]. PCR products were purified by using the QIAquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif.) as described by the manufacturer. The *Taq* Dyedeoxy

Terminator Cycle Sequencing kit (Applied Biosystems, Foster, Calif.) was used to directly sequence the PCR products according to the protocol provided by the manufacturer. The sequencing primers have been described previously [20]. The sequence reaction mixtures were electrophoresed on an Applied Biosystems 373S DNA sequencer.

Phylogenetic analyses of 16S rRNA gene sequence data. The sequences were loaded into the 16S rRNA sequence data base of the Technical University of Munich using the program package ARB. The tool ARB_ALIGN was used for sequence alignment. The alignment was visually inspected and corrected manually. Tree topologies were evaluated by performing maximum parsimony, neighbour joining, and maximum likelihood analysis with different sets of filters. Only sequences with at least 1200 nucleotides were used for the calculation of different trees. The partial sequence of strain I was added to the reconstructed tree by applying parsimony criteria without allowing changes in the overall

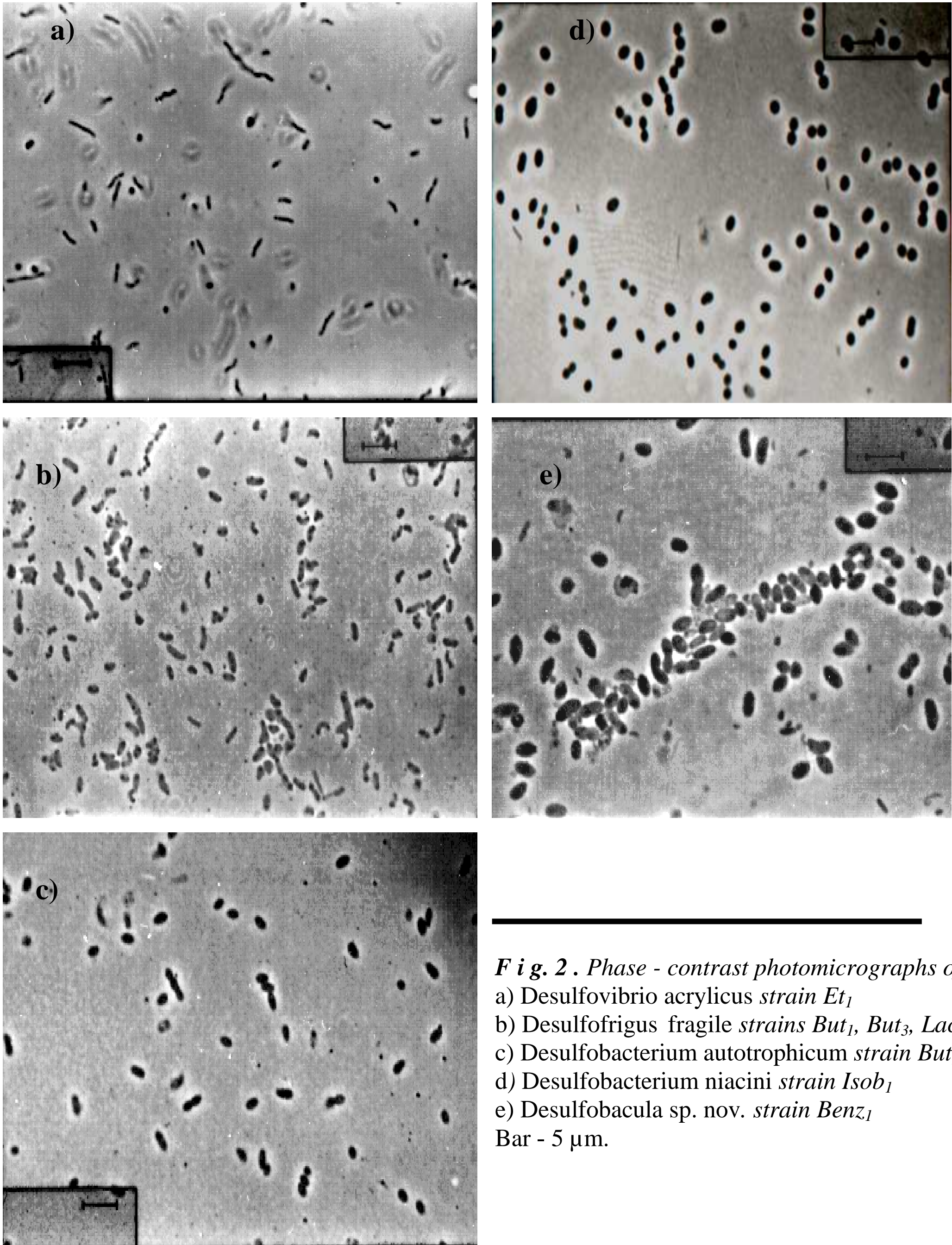
tree topology. The strain designations and nucleotide sequence accession numbers which were not included in the ARB database are as follows: *Desulfobacula toluolica*^{T} DSM 7467, X70593; *Desulfobacula phenolica*^{T} DSM 3384, AJ237606; *Desulfospira joergensenii*^{T} DSM 10085, X99637; *Desulfotignum balticum* (strain Sax), DSM 7044, AF233370; *Desulfobacterium autotrophicum* DSM 3382^{T3}, HRM$_2$; *Desulfobacterium niacini* DSM 2650^{T4}, *Desulfofrigus fragile,* DSM 2345^{T2}, LS$_v$21; *Desulfovibrio acrylicus* DSM 10141 ^{T1},W218.

Results. *Physiological and morphological properties.* Using the organic compounds as described above seven mesophilic sulphate-reducing bacteria were isolated: Et$_1$, But$_1$, But$_2$, But$_3$, Lac$_1$, Isob$_1$, Benz$_1$. Morphological caracterisation of the isolated strains determined by light microscopy revealed that all strains were motile, single or in chain formed oval to vibrio-, rod-shaped cells (Fig. 2). Three strains (But1, But3, Lac1) were morphologically and nutritionally similar. All sulphate-reducers were mesophilic, Gram-negative. The physiological and morphological properties of the isolated strains are listed in Table1.

A significant production of H_2S by sulphate reduction was observed after 2-3 weeks of incubation (at 28 °C in the dark) with acetate, propionate, lactate, butyrate and benzoate as an electron donor and carbon source. For the other five compounds sulphide production occurred after about 1 month. During isolation and cultivation best growth was observed on acetate, propionate, hexadecanoate, benzoate, butyrate, lactate, ethanol (Table 2).

Phylogenetic analysis. The phylogenetic affiliation of the isolated SRB was determined according to a partial 16S rDNA sequencing. They were most closely affiliated with the genera *Desulfovibrio*, *Desulfofrigus*, *Desulfobacterium* and *Desulfobacula* (1, 3, 2 and 1 strain, respectively).

The phylogenetic affiliation of the seven isolated strains according to their partial 16S rDNA sequence and phylogenetic position is shown in Table 3 and Fig. 3.



F i g. 2 . *Phase - contrast photomicrographs of*
a) Desulfovibrio acrylicus *strain* Et_1
b) Desulfofrigus fragile *strains* But_1, But_3, Lac_1
c) Desulfobacterium autotrophicum *strain* But_2
d*)* Desulfobacterium niacini *strain* $Isob_1$
e) Desulfobacula sp. nov. *strain* $Benz_1$
Bar - 5 µm.

Table 1

Morphological and physiological properties of sulphate-reducing bacteria isolated from MPN dilutions of the bottom sediments

Characteristic	*Desulfovibrio acrylicus* (strain Et_1)	*Desulfofrigus fragile* (strain But_1)	*Desulfobacter autotrophicum* (strain But_2)	*Desulfofrigus fragile* (strain But_3)	*Desulfofrigus fragile* (strain Lac_1)	*Desulfobacter niacini* (strain $Isob_1$)	*Desulfobacula sp. nov.* (strain $Benz_1$)
Morphology	Spiral to vibrioid-shaped cell	Rod-shaped cell with PHB inclusions	Oval shaped cell; single or chain of cells	Rod-shaped cell with PHB inclusions	Rod-shaped cell with PHB inclusions	Oval or almost coccoid cells	Oval to curved rod-shaped cell
Width x length (µm)	0.5 -1.3 x 0.8 - 5	0.8 x 3.2-4.2	0.7-1.3 x 1.5 –2.8	0.8 x 3.2-4.2	0.8 x 3.2-4.2	1.5 x 3	1-1.5 x 2 –3
Optimal temperature (°C)	28 - 30	18 - 27	20 - 26	18 - 27	18 - 27	29	28
Motility	+	+	+	+	+	+	+
Electron donors:							
H_2/CO_2	-	-	+*	-	-	-	-
Formate	-	+	-	-	-	-	-
Propionate	-	+	-	-	+	-	-
Acetate	-	+	+	+	+	-	+
Butyrate	-	+	+	+	-	-	+
Isobutyrate	-	-	-	-	+	+	-
Fatty acid : C atoms	-	4, 10, 12	4, 10, 16	4, 12	4	4, 10, 12	4
Ethanol	+	+	-	-	-	+	+
Lactate	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	+
Fumarate	+	+	+	+	+	-	+
Succinate	+	+	-	-	-	+	+
Malate	-	-	-	-	-	-	-
Benzoate	-	-	+	+	-	+	+
Glycerol	+	+	+	+	+	+	+
Butanol	+	+	+	+	-	+	+
Fermentative growth on:	pyruvate	pyruvate	pyruvate	pyruvate	pyruvate	pyruvate	pyruvate
Electron acceptors:							
Sulphate (28 mM)	+	+	+	+	+	+	+
Sulphite	-	-	+	+	-	+	+
Growth factor requirement	vitamins	vitamins	vitamins	vitamins	vitamins	vitamins	vitamins

* -autotrophic growth; + - good growth; - no growth.

Table 2

Phylogenetic affiliation of the 7 isolates according to their partial 16S rDNA sequence (primer GM3F: 5’- AGAGTTTGAT Ca/c TGGC - 3’ and GM4R: 3’-TCCAGCATTGTTCCAT-5’)

Isolate	Close relative from databank	% similarity	Genbank accession no.
Et_1	*Desulfovibrio acrylicus* DSM 10141 [T1)]	98	**W218**
But_1	*Desulfofrigus fragile* DSM 12345 [T2)]	97.9	LS_v21
But_2	*Desulfobacterium autotrophicum* DSM 3382[T3)]	99	HRM_2
But_3	*Desulfofrigus fragile* DSM 12345 [T2)]	98	LS_v21
Lac_1	*Desulfofrigus fragile* DSM 12345 [T2)]	98	LS_v21
$Isob_1$	*Desulfobacterium niacinii* DSM 2650[T4)]	99	-
$Benz_1$	*Desulfobacula toluolica* DSM 7467[T5)]	96	X70593

1) v a n d e r M a a r e l *et al.* [28]
2) K n o b l a u c h *et al.* [19]
3) B r y s c h *et al.* [6]
4) I m h o f f and P f e n n i g [17]
5) R a b u s *et al.* [25]

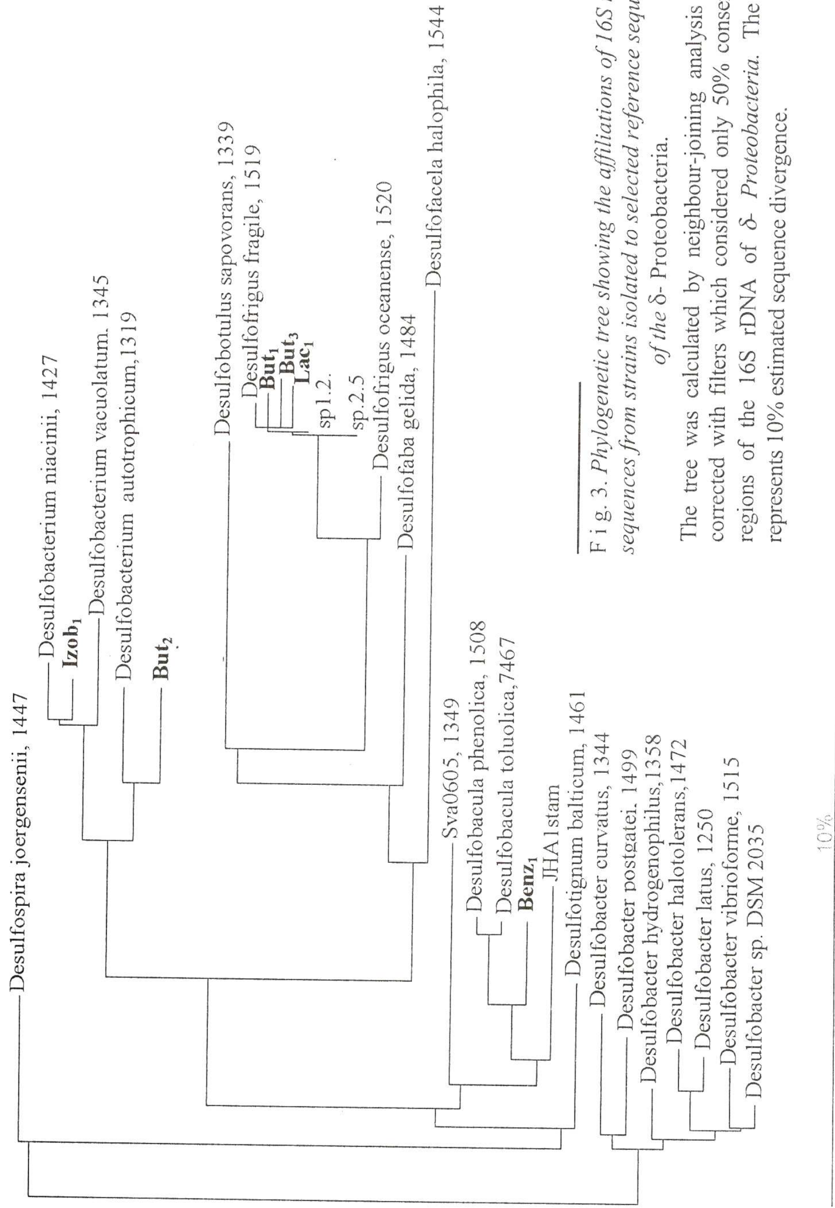
Table 3

MPN counts of sulphate-reducing bacteria with different substrates in sediment samples

SUBSTRATE	MPN / G WET SEDIMENT
Acetate (10 mM)	0.24 x 10^6
Lactate (10 mM	0.17 x 10^4
Butyrate (10 mM)	0.28 x 10^4
Benzoate (2 mM)	0.14 x 10^5
Ethanol (10 mM)	0.14 x 10^4
Propionate (10 mM	0.14 x 10^6
Formate (10 mM)	0.072 x 10^3
Hexadecanoate (1 mM)	0.17 x 10^6

Discussion. In marine ecosystems, particularly in coastal zones and estuaries, sulphate-reducing bacteria play a key role in the sulphur cycle and organic matter decomposition [14, 15]. Marine bottom sediments provide an optimum environment for these microorganisms.

All strains isolated in this study were mesophilic, dissimilatory sulphate-reducing bacteria. Surprinsingly, we isolated from this coastal temperate sediment the species *Desulfofrigus fragile.* This bacterium, isolated initial by v a n d e r M a a r e l *et al.*[28] from an artic sediment, has the ability to grow at 4°C. Its presence in our sampling site could be an adaptation to changing environmental conditions and this strain seems to be psychrotolerant, not psychrophilic.



F i g. 3. *Phylogenetic tree showing the affiliations of 16S rDNA sequences from strains isolated to selected reference sequences of the* δ- Proteobacteria.

The tree was calculated by neighbour-joining analysis and corrected with filters which considered only 50% conserved regions of the 16S rDNA of δ- *Proteobacteria.* The bar represents 10% estimated sequence divergence.

SRB numbers in the bottom sediments of the study area (East Constanßa, 50 m deep) ranged between 0.72×10^4 and 0.24×10^6 cells per g wet sediment. Table 2 illustrates the numbers of viable sulphate-reducing bacteria incubated on different organic substrates as sources of electrons and of energy and carbon in sulphate reduction. The MPN dilution series in this study were incubated with eight different substrates to encompass a broad variety of SRB populations. Some of the most common fermentation products in marine sediments, such as acetate, propionate, butyrate and lactate were used. All these substrates are known to be used by pure cultures of SRB [30]. The data show that SRB inhabiting the bottom sediments of this particular area of the Romanian Black Sea coast were able to use all organic substrates as electron donors and as carbon and energy sources. At the same time, it was found that the different physiological groups of SRB inhabiting the bottom sediment preferred various forms of organic carbon. Acetate-utilising bacteria were present in the greatest number (0.24×10^6 MPN/g sediment). Propionate and hexadecanoate were also optimum substrates for the SRB in sediments, while lactate-, ethanol-, formate- and butyrate-utilising bacteria were present in lower numbers (Table 2). Variations in these physiological groups of SRB indicate a significant heterogeneity of this bottom sediment.

Our preliminary study showed high numbers of anaerobic SRB in the upper layer of sediment from the continental shelf of the Black Sea. The presence of these bacteria in the Romanian continental shelf of Black Sea (50 m depth) can be explained as a consequence of eutrophication. During recent decades, the Black Sea has become seriously influenced by anthropogenic sources. Large quantities of inorganic and organic compounds have been introduced by rivers and by industrial and domestic discharges. A lot of organic material entered in the sediment. The final mineralisation of carbon might occur in the sediment and not in the water column. Our results demonstrated that SRB were important for carbon mineralisation in the sediment from the Black Sea shelf area, leading to formation of hydrogen sulphide in the shelf bottom waters which resulted in hypoxia and, occasionally, in anoxia.

Conclusions. 1. Our attempts to enrich and cultivate the SRB from the Romanian Black Sea coast were successful.

2. Seven strains of SRB were isolated from coastal sediments (Romanian Black Sea sector) and identified as *Desulfofrigus fragile, Desulfobacterium autotrophicum, Desulfobacterium niacini, Desulfovibrio acrylicus* using molecular techniques.

3. An unexpected result of this study was the isolation in pure culture of a novel marine sulphate-reducing bacterium belonging to the genus *Desulfobacula*.

4. Our preliminary study showed high numbers of anaerobic SRB in the upper layer of sediment from the continental shelf of Black Sea. The results presented in this paper may contribute to the explanation of the role of SRB in the process of organic matter destruction in the bottom sediments of the Romanian Black Sea sector and formation of hydrogen sulphide in the shelf bottom waters which results in hypoxia or, occasionally, in anoxia.

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RECENZII - BOOK REVIEWS

Mirco Grimm, Robert Jones and Luca Montanarella, **Soil Erosion Risk in Europe**, European Commission, Joint Research Centre, Institute for Environment and Sustainability, European Soil Bureau, Ispra (Varese), Italy, 2002, IV + 40 pages (of A4 format), including 18 figures and 3 tables.

The volume is structured into Contants, Summary, Introduction, chapters entitled "Processes of soil erosion", "Assessing soil erosion risk", "Indicators of soil erosion", Conclusions an recommendations, References and Glossary.

It is well documented in the volume that soil erosion by water is a widespread problem throughout Europe. Thus, water erosion causing loss of topsoil and terrain deformation affects 52.3% of soils in Europe, including the European part of the former Soviet Union.

A detailed description is devoted to soil erosion risk assessment. Seven recent approaches to this assessment are comprehensively charactherised and advantages and limitations of each approach are specified.

It is emphasised in the Conclusions and recommendations that soil erosion, being a complex problem, requires a mul tidisciplinary approach. Therefore, soil scientists must work increasingly with scientists from other disciplines, for example biologists, geologists, chemists, mathematicians, statisticians, ecologists, social scientists and economists to address the problem of soil erosion. We should like to add to this recommendation that a stronger collaboration among specialists in different domains of soil science (soil biologists including soil microbiologists, soil biochemists including soil enzymologists, soil chemists and soil physisists) is also needed. Our recommendation is supported even by the chapter "Indicators of soil erosion", in which there is no word on the microbial and enzymatic indicators of the soil erodibility and of the efficiency of the measures taken for rehabilitation of eroded soils. We mention here that results of the first enzymological study of the soil covering a hillslope prone to erosion were published nearly 50 years ago in Germany (Koepf, 1054).

Soil Erosion Risk in Europe is a valuable source of information for all scientists, technologists and decision makers interested in preventing and combating soil erosion in Europe and elsewhere.

STEFAN KISS

G.A. Evdokimova, I.V. Zenkova, V.N. Pereverzev, **Biodinamika protsessov transformatsii organicheskogo veshchestva v pochvakh Severnoi Fennoskandii** *(Biodynamics of the Transformation Processes of Organic Substance in Soils of Northern Fennoscandia),* Kol'skii Nauchnyi Tsentr, Rossiiskaya Akademiya Nauk (Kola Science Centre, Russian Academy of Sciences), Apatity, 2002, 154 pages, including 41 figures and 39 tables in the text.

The book is structured into Introduction; Chapter 1, "Conditions of the investigations" (Characteristics of the climate; Meteorological conditions in the years of the investigations; Objects and methods of the investigations); Chapter 2, "Characteristics of soils" (Podzolic soils: Content of heavy metals in podzolic soils; Peat soils); Chapter 3, "Anthropogenic impact on the biot of soils" (Industrial impact on microorganisms; Agricultural impact on micro-

organisms; Fauna of invertebrates in natural podzols; Industrial impact on invertebrates); Chapter 4, "Transformation of plant resudues in soil" (Intensity of the decomposition of plant residues; Changes in the chemical composition of plant residues during their decomposition; Humus substances of the decomposing plant residues; Microorganism of plant residues in forest podzols; Microorganisms of plant residues in cultivated soils; Invertebrates of plant residues in forest podzols); Chapter 5, "Natural and antropogenic peculiarities of the interaction between soil microorganisms and invertebrates during transformation of plant residues in soils of Northern Fennoscandia"; Conclusions; references (177 papers cited); Appendices (7 tables); Contents.

The investigations described in the book were carried out during the vegetation period of three years (1997-1999). Experimental plots were installed in the Murmansk region (Kola peninsula), namely in the vicinity of the cities of Apatity (plots 1 and 2) and Moncheegorsk (plot 3) and in Northern Norway, namely on the territory of the Experimental Centre Svanhovd (plots 4-6) and in the vicinity of the city of Sibotn, Troms region (plots 7 and 8).

Decomposition of plant residues was studied on each plot. in addition, the impact of pollutants emitted from nonferrous metallurgical plants was also assessed, on plots 3 and 4, using the unpolluted plot 7 for comparison.

The results obtained are valuable contributions to a better understanding of the processes of the decomposition of plant residues under different climatic conditions and of the impact of heavy metal pollution on these processes.

The book constitutes a useful source of information for students and specialists in soil micronbiology, zoology, biochemistry and chemistry as well as for scientists, technologists and decision makers interested in studying and preventing environmetntal pollution.

STEFAN KISS