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FAUNISTIC AND ECOLOGICAL RESEARCHES ON THE TERRESTRIAL ISOPODS FROM THE SUPERIOR SECTOR OF THE ARIEŞ RIVER BASIN

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SUMMARY. – In the natural ecosystems from the superior sector of the Arieş River basin (spruce forests, mixed forests, beech forests, coppices and meadows) there are 8 species of terrestrial isopods: *Ligidium hypnorum, Hyloniscus riparius, Hyloniscus transsylvanicus, Protracheoniscus politus, Megepimerio vareae, Trachelipus wächtleri, Porcellium conspersum* and *Armadillidium carniolense.* The specific structure of the isopod communities differs in relation to the type of ecosystem, and also between ecosystems of the same type. We can not conclude that a certain isopod community is typical for a certain ecosystem from low mountain areas. In the researched mountain meadows there live sylvan, humicolous and paludicolous isopod species. The individuals of the populations have a dispersion that is limited to the microhabitats with high humidity and moderate temperatures. Here there are no praticolous species, which are typical for the meadows occurring on hilly and plane areas. Probably, the average annual temperatures recorded in mountain meadows are situated outside of the limits of the optimum thermal values for praticolous species. In forests, the sylvan species *Protracheoniscus politus* and *Trachelipus wächtleri* have a wide dispersion on the surface of the soil underneath the litter layer, due to the broad limits of their ecological valence. They tolerate wide oscillations of the temperature and humidity. Paludicolous species (*Ligidium hypnorum, Hyloniscus transsylvanicus, H. riparius*), humicolous species (*Porcellium conspersum*) and two sylvan species (*Megepimerio vareae* and *Armadillidium carniolense*) have a very low tolerance to the variations of temperature and humidity. Their dispersion in a biotope is limited to microhabitats with high and constant humidity and moderate temperatures that can not exceed 15-18ºC. The dispersion pattern influences the values of the frequency of the species, and also their numerical and relative abundance. The species that depend on a certain type of microhabitat have, in general, small populations in an ecosystem and a low frequency in the collected samples. However, these are not considered to be accidental species, as long as their favorite microhabitats are permanent in an ecosystem. They belong to the category of accessory species. The populations of *Protracheoniscus politus* have a frequency of 70-100% in all the studied ecosystems. It is a constant and euconstant species, both in forest ecosystems and in meadows. In the forest ecosystems, it is a dominant species. In the studied ecosystems in which the values of ecological diversity and evenness are relatively high, for instance in certain spruce and beech forests, the isopod populations are numerically reduced for all species. The ecological conditions are at the limit of their biological optimum and, in consequence, the survival coefficient is low for the entire community of species.

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At present, the study of terrestrial isopods on an international level refers to aspects that are less known, for example: the species' preference for certain habitats, food and feeding behaviour, population dynamics and biological cycle [2, 4, 6, 7, 10]. Research on the preference of isopods for certain habitats was conducted for few species, for example for *Armadillidium vulgare*, *Trichoniscus pussilus* and *Philoscia muscorum* [9]. A preferential behaviour for microhabitats was also observed in aquatic species [1, 5, 7, 8]. In our research we have considered especially the ecological aspects of the communities of terrestrial isopods in the Arieş basin. In this article we present the results that were obtained in the superior sector of the Arieş basin.

The superior sector of the Arieş basin is located in a low mountain area, and it is characterised by great ecological diversity. Forests are the predominant ecosystems (spruce forests, mixed forests, beech, deciduous tree forests, coppices). There also are open ecosystems (meadows, rocky areas). The ecological diversity is also accentuated by the inclination and aspect of the slopes, the thickness of the soil layer, the network of springs and the depth of the ground water. Between the years 1998 and 2001 we studied the communities of terrestrial isopods from the natural ecosystems located in the upper sector of the Arieş basin. We conducted research in five types of ecosystems: spruce forests, mixed forests (spruce and beech), beech forests, coppices and meadows.

Spruce forests – are dominant in the area. They are different mainly due to the age and density of the trees, the landscape of the area, the hydrographic network (springs and swampy areas), herbaceous vegetation, shrubby vegetation, the thickness of the soil and of the litter layer etc. The spruce forests in the studied area belong to two important types of plant associations: *Sphagno – Piceetum* (spruce forests with high humidity) and *Oxalo – Piceetum* (spruce forests with moderate humidity).

Mixed forests (spruce and beech) – are old forests, the trees being over 80 years old. The herbaceous vegetation is very reduced; the litter layer is thick and continuous. The humidity of the soil is low during summer, when the soil becomes arid. The soil layer is interrupted by surfacing rock fragments that are covered with moss. These are preferred microhabitats for certain isopod species [11].

Coppices – located on the banks of the Arieşul Mare and Arieşul Mic Rivers, consist of alder trees, willows and rare shrubs. The soil is alluvial and has high humidity, and the herbaceous layer is abundant. The litter layer is thin and discontinuous, due to the fast decomposition of the willow and alder leaves.

Meadows – are located on slopes with different inclinations and aspects. The insolation at the surface of the soil is very strong, causing the rapid evaporation of the ground water. Due to the very rich hydrographic network, in some meadows there are small swampy areas, with very humid soil. The isopod species that live here are paludicolous, and sometimes there also are sylvan species, but with populations that are numerically reduced. On the surface of the meadows there are rare and isolated shrubs (hazel, *Rosa canina*), and also isolated trees.

Material and methods. In the studied area we have collected samples in 16 stations located in different points (Fig. 1). In each ecosystem we placed, at the beginning of June, 5-10 Barber traps with 4% formaldehyde. The captured animals were collected in July. We collected a total number of 3940 individuals. For the data processing we used ecological indices: numerical abundance, relative abundance, frequency, ecological diversity and evenness, and ecological similarity.

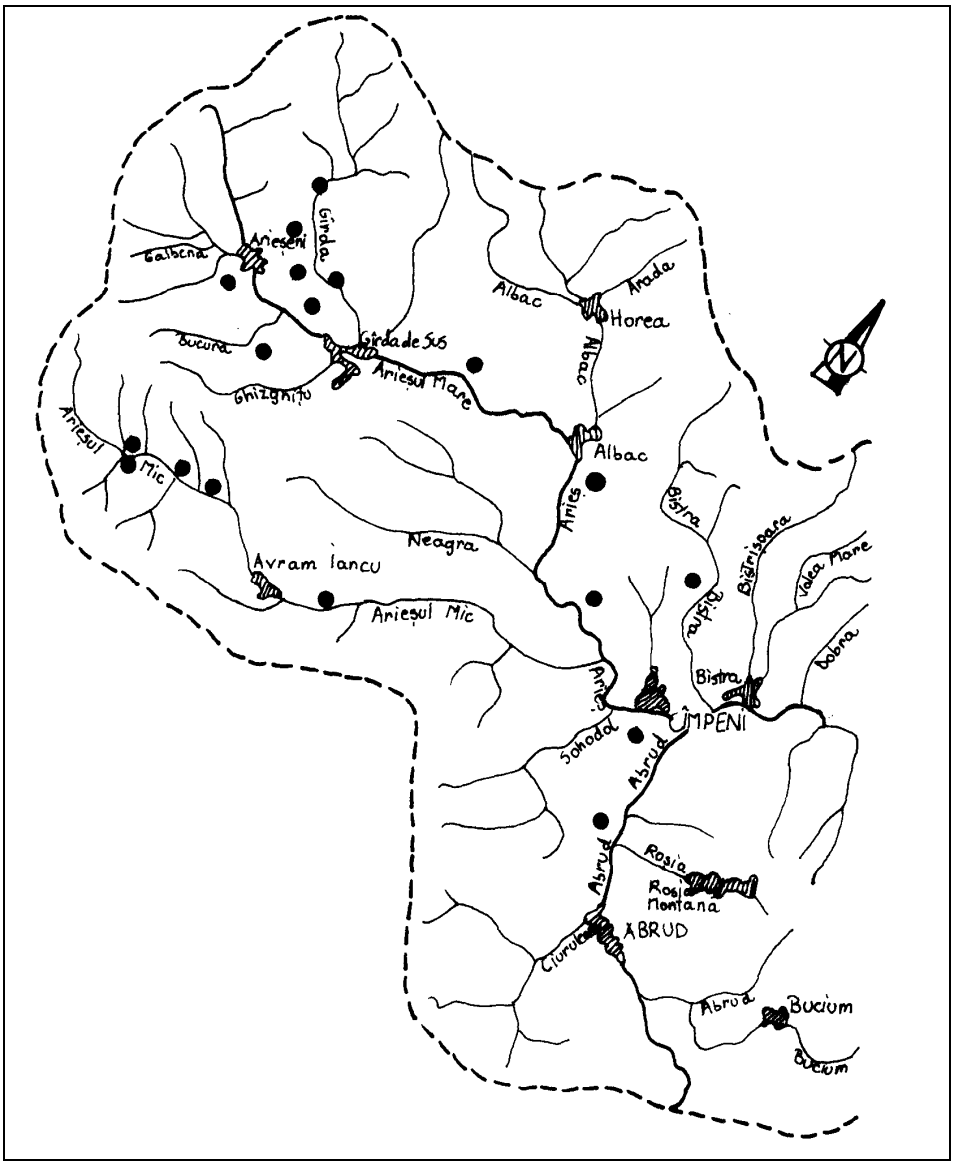

Fig. 1. *The superior sector of the Arieş basin with the sampling sites.*

Results and discussion. In the collected material, we identified eight species of terrestrial isopods (Tables 1 and 4). Three species are paludicolous (*Ligidium hypnorum, Hyloniscus transsylvanicus, H. riparius*), four are sylvan species (*Protracheoniscus politus, Megepimerio vareae, Trachelipus wächtleri, Armadillidium carniolense*) and one is humicolous (*Porcellium conspersum*). Their distribution in the ecosystems from the studied area and the numeric abundance of the populations are different, in relation to the ecological particularities of each type of ecosystem. There are notable differences between forests of the same type, for example between spruce forests (Table 1), thus it can not be asserted that a certain community of species is characteristic to all spruce forests. Of the five spruce forests that were studied, only in two the isopod communities consist of the same species (4 species), but the numerical and relative abundance of the isopod populations differs greatly. Three species of isopods (*Hyloniscus transsylvanicus, Protracheoniscus politus* and *Trachelipus wächtleri*) are found in all of the five spruce forests. *Ligidium hypnorum* lives in four spruce forests, and each of the other species lives only in one spruce forest, with numerically reduced populations (Table 1). It is a clear proof that these species have ecological valences with much narrower limits compared to the species that have a higher distribution in this area. Their presence in only one spruce forest can be explained by their dispersion only in a certain type of microhabitat from the perimeter of the biotope, where they find optimum life conditions.

Table 1

Numerical abundance of terrestrial isopod communities in the spruce forests located in the superior sector of the Arieș basin (0/trap)

No.	Species	Arieșeni				Gârda
		1	**2**	**3**	**4**	**5**
1	*Ligidium hypnorum*	7	5		3	1
2	*Hyloniscus riparius*				2	
3	*Hyloniscus transsylvanicus*	6	7	13	12	5
4	*Protracheoniscus politus*	10	106	206	189	3
5	*Porcellium conspersum*				1	
6	*Megepimerio vareae*				1	
7	*Trachelipus wächtleri*	1	5	1	5	1
8	*Armadillidium carniolense*	1				
	Total no. of individuals	124	486	658	419	44
	Total no. of species	5	4	3	7	4

1 - Spruce forest, soil with high humidity.
2 - Spruce forest, soil with moderate humidity.
3 - Spruce forest, slope with eastern aspect.
4 - Spruce forest, slope with western aspect.
5 - Spruce forest, soil with moderate humidity.

The existence of significant differences regarding the structure of the isopod communities in the five spruce forests that were studied can also be observed looking at the values of the Morisita ecological similarity index (Table 2). Communities with resembling specific structure are found in the forests that are situated on a slope with eastern aspect, and the other on a slope with western aspect. In the other spruce forests, the structure of the isopod communities differs greatly. The isopod fauna in the spruce forest also differs in what concerns the size of the populations. The values of the numerical abundance (Table 1) show that the most numerous populations are those of the species *Protracheoniscus politus*, in the majority of the spruce forests. Relatively high populations were also registered for *Hyloniscus transsylvanicus*. For the five spruce forests we also calculated the ecological diversity index and evenness (Table 3); higher values were recorded in the spruce forest with very humid soil where five species of isopods live, and in the spruce forest that has a soil with moderate humidity, with four isopod species. Here, the numerical differences between the populations of the isopod species are much lower. On the other hand, the ecological diversity and evenness values are much lower for other three spruce forests, where the populations of *Protracheoniscus politus* are very abundant, compared to the populations of the other species. The size of the populations of the isopod communities in the studied forests shows to what extent the ecological conditions meet the ecological optimum of the species.

In Table 4 we present the isopod communities from all types of ecosystems that belong to the studied area. We summed up the data from several ecosystems of the same type, in order to observe the overall resemblances and differences between the different types of ecosystems. The table also contains the values of the numerical abundance, relative abundance and the frequency of the species.

Most of the isopod species live in spruce forests (8 species) and in coppices (7 species), and the fewest live in the mixed forests (spruce and beech) (3 species).

It is important to emphasise the resemblance of the isopod communities from the mountain meadows in the Arieş area with the isopod communities from forests. In the meadows from the studied area there are species that live in the forests (sylvan and paludicolous species). The average thermal values, lower in the meadows from the mountain areas, as well as the numerous springs, the small depth of the ground water, favour the existence of very humid microhabitats with abundant herbaceous vegetation, where sylvan and paludicolous isopod species were found. The dispersion of the individuals of all species is, however, limited to these microhabitats. The isopod species that lack here are the praticolous ones (*Trachelipus nodulosus, Porcellio scaber, Armadillidium vulgare* etc.), which are characteristic to the above-ground fauna from the meadows, hilly areas and planes. Similar results were also recorded in mountain meadows in the Dorna Depression [3]. We consider that the values of the average annual temperatures in the mountain meadows are outside the limits of the thermal optimum of praticolous species.

Table 2

The values of the Morisita index of ecological similarity between different spruce forests from the superior sector of the Arieş basin

Ecosystem	1	2	3	4	5
1		0.0666	0.0408	0.0518	0.6983
2			0.9957	0.9993	0.5561
3				0.9991	0.5363
4					0.5453
5					

1 - Spruce forest, soil with high humidity.

2 - Spruce forest, soil with moderate humidity.

3 - Spruce forest, slope with eastern aspect.

4 - Spruce forest, slope with western aspect.

5 - Spruce forest, soil with moderate humidity.

Table 3

The values of the Shannon - Wiener ecological diversity index and evenness in the spruce forests from the superior sector of the Arieş basin

	Spruce forests				
	1	**2**	**3**	**4**	**5**
H'	0.5543	0.2310	0.1047	0.1963	0.4156
e	0.7930	0.3837	0.2195	0.2323	0.6904
No. of species	5	4	3	7	4

1 - Spruce forest, soil with high humidity.

2 - Spruce forest, soil with moderate humidity.

3 - Spruce forest, slope with eastern aspect.

4 - Spruce forest, slope with western aspect.

5 - Spruce forest, soil with moderate humidity.

The Morisita ecological similarity index shows smaller differences between different types of ecosystems (Table 5), compared to the ecosystems that belong to the same type, such as spruce forests (Table 2). By considering the data from several ecosystems of the same type, the differences between different types of ecosystems are reduced. In Table 5, the values of the similarity index are based on the summation of the data from all the studied spruce forests, mixed forests, beech forests, coppices and meadows.

Table 4

Numerical and relative abundance and frequency of the terrestrial isopod communities in the natural ecosystems located in the superior sector of the Arieș basin

No.	Species	I. Spruce forest				II. Spruce and beech forest				III. Beech forest				IV. Coppice				V. Meadow			
		no.ind.	x̄/trap	%	F	no.ind.	x̄/trap	%	F	no.ind.	x̄/trap	%	F	no.ind.	x̄/trap	%	F	no.ind.	x̄/trap	%	F
1	*Ligidium hypnorum*	60	3.15	3.46	33.30				7.70	95	6.33	10.40	40.00	13	1.85	4.54	60.00	9	0.69	1.60	46.00
2	*Hyloniscus riparius*	3	0.17	0.17	6.60					5	0.33	0.54	20.00	21	3.00	7.34	80.00	7	0.53	1.24	7.50
3	*Hyloniscus transsylvanicus*	144	7.57	8.31	66.60	6	0.60	3.94	46.10	59	3.93	6.46	26.60	64	9.14	22.37	40.00	126	9.69	22.45	46.00
4	*Protracheoniscus politus*	1484	78.10	85.73	93.00	137	13.70	90.13	61.50	723	48.20	79.27	86.70	159	22.70	55.60	100.00	367	28.20	65.40	92.00
5	*Porcellium conspersum*	1	0.05	0.05	6.00				7.00	1	0.06	0.10	6.60	2	0.28	0.70	40.00	6	0.46	1.06	15.70
6	*Megepimerio varese*	1	0.05	0.05	6.00																
7	*Trachelipus wächtleri*	36	2.07	2.07	46.70	9	0.90	5.29	38.50	29	1.93	3.17	46.60	26	3.71	9.09	80.00	46	3.53	8.19	53.70
8	*Armadillidium carniolense*	2	0.11	0.11	6.00									1	0.14	0.34	20.00				
	Total no. of individuals	1731				152				912				286				561			
	Total no. of species	8				3				6				7				6			

% - Relative abundance.
F - Frequency.

Table 5

The values of the Morisita index of ecological similarity between different types of ecosystems from the studied area

	Spruce	Mixed forest	Beech	Coppice	Meadow
Spruce		0.9966	0.9935	0.8938	0.9474
Mixed forest			0.98462	0.8667	0.9275
Beech				0.9110	0.9522
Coppice					0.9869
Meadow					

In the studied ecosystems, the values of the numerical abundance differ from one species to another, as well as from one type of ecosystem to another (Table 4). In all types of ecosystems, the highest values of the numerical abundance were recorded for *Protracheoniscus politus.* Higher values were also recorded for some populations of *Hyloniscus transsylvanicus* in spruce forests, coppices and meadows, for *Ligidium hypnorum* in spruce forests and in the beach forest, and for *Trachelipus wächtleri* in spruce forests and coppices. For the other species, the recorded values were low, indicating the existence of numerically reduced populations. The same aspect can also be observed by analysing the values of the relative abundance. In the isopod communities from all the studied ecosystems, the numerically dominant populations are those of *Protracheoniscus politus*, a species that is dominant in forests, and in the meadows from mountain areas. The existence of numerically reduced populations for certain isopod species, such as *Porcellium conspersum, Megepimerio vareae, Armadillidium carniolense*, can be explained by the very narrow limits of their ecological valence, a trait that limits their dispersion only to microhabitats with optimum life conditions.

The values of the frequency of the species in the types of studied ecosystems are variable (Table 4). *Protracheoniscus politus* is constant in mixed forests, spruce forests and beech forests, and euconstant in the other ecosystems. *Ligidium hypnorum* is constant in coppices, accessory in spruce forests, beech forests and mountain meadows, and accidental in mixed forests. Other euconstant and constant species are: *Hyloniscus riparius* in coppices, *H. transsylvanicus* in certain spruce forests, *Trachelipus wächtleri* in coppices and mountain meadows. *Porcellium conspersum, Megepimerio vareae* and *Armadillidium carniolense* are considered to be accessory species in all types of ecosystems. Their sporadic presence in the samples is, however, explained by the dispersion of individuals, which is limited only to certain microhabitats with optimum ecological conditions. In this case, the frequency of the species in an ecosystem and the size of the populations depend on the frequency of such microhabitats. These isopod species can be included in the category of

accessory species, even if the values of their frequency are low and very low. Their sporadic presence in the samples collected from an ecosystem is not determined by the incidental immigration of some individuals from neighbouring ecosystems, but by the small number of microhabitats that they populate. For the isopods, as well as probably for other groups of animals that have reduced mobility, certain species can accidentally appear in an ecosystem if, unintentionally, a number of individuals were brought in by man, while conducting certain activities in the area. The frequency of the species and the size of the populations also depend on the dispersion of the individuals in a biotope. The isopod species that are constant and euconstant have a wider dispersion on the surface of the biotope of an ecosystem. In forests, *Protracheoniscus politus* and, in some cases, *Trachelipus wächtleri* have such dispersion. They live under the litter layer and have a higher tolerance to the oscillation of the ecological factors. The aggregation behaviour of the individuals is much more reduced compared to paludicolous, corticolous, petrophilous and synanthrope species. As a consequence, their frequency is higher in the samples, and their populations are more numerous.

The ecological diversity and evenness were also computed for different types of ecosystems. In this case, the ecological diversity and evenness indices have small values (Table 6). Higher values for these indices were recorded for meadows, but here, as well, with the exception of species *Protracheoniscus politus*, the populations are more reduced numerically, although only six isopod species live here.

In all the studied ecosystems the populations of *Protracheoniscus politus* are numerically dominant. The results of our research lead to the conclusion that the values of the ecological diversity and evenness indices of the isopod communities are determined to a greater extent by the limits of their ecological valence, which influences the size of the populations and their dispersion on the surface of a biotope, and less by the number of species in a biocoenosis.

Table 6

The values of the Shannon - Wiener ecological diversity index and evenness in different types of ecosystems from the studied area

	Spruce	Mixed forest	Beech	Coppice	Meadow
H'	0.2446	0.1687	0.3224	0.5498	0.4289
e	0.2709	0.3537	0.4144	0.6506	0.5512
No. of species	8	3	6	7	6

In forest ecosystems, the isopods do not present intraspecific or interspecific competition in their ecological niches. As a consequence, there is no inverse relation between the number of species that form a community in an ecosystem and the size

of the populations. The size of the populations is influenced by the mortality rate at juvenile stages, which depends on the tolerance of the species to the values and fluctuations of the ecological factors, especially temperature and humidity.

Conclusions. 1. In the natural ecosystems from the superior sector of the Arieş basin (spruce forests, mixed forests, beech forests, coppices and meadows) there are eight species of terrestrial isopods that live there, of which three species are paludicolous (*Ligidium hypnorum, Hyloniscus transsylvanicus, H. riparius*), four species are sylvan (*Protracheoniscus politus, Megepimerio vareae, Trachelipus wächtleri, Armadillidium carniolense*), and one species is humicolous (*Porcellium conspersum*).

2. The communities of terrestrial isopods from the ecosystems differ in terms of their specific structure, numerical abundance and relative abundance. The differences are greater even in the case of ecosystems that belong to the same type, thus it can not be concluded that a certain isopod community is characteristic to a certain type of ecosystem.

3. In the meadows from the superior sector of the Arieş basin (low mountain area), the isopod communities consist of sylvan, paludicolous and humicolous species, that are characteristic to forest type ecosystems. Praticolous species, that are characteristic to meadows from hilly areas and planes, are absent.

4. In forest type ecosystems, sylvan species of isopods (*Protracheoniscus politus, Trachelipus wächtleri*) have a wide dispersion on the surface of the soil, under the litter layer. Paludicolous and humicolous species have a limited dispersion on the surfaces of the microhabitats that have optimum ecological conditions (especially very high humidity and moderate temperatures). The presence of these species in an ecosystem depends on the presence of such microhabitats in the perimeter of the biotope.

5. In mountain meadows, both paludicolous and sylvan species have a dispersion that is limited to moist microhabitats, located around springs or where the ground water is near the surface of the soil. *Protracheoniscus politus* populations are more abundant in meadows as well, compared to the populations of the other species.

6. In all the studied ecosystems, *Protracheoniscus politus* populations are numerically dominant and have a frequency of more than 70% in the samples (constant and euconstant). *Porcellium conspersum, Megepimerio vareae* and *Armadillidium carniolense* are present only in some ecosystems, with numerically reduced populations, their presence in an ecosystem depending on the presence of preferred microhabitats. They are included in the category of accessory species. Paludicolous species are constant in some ecosystems (coppices, spruce forests, meadows), and in others appear as accessory species.

7. The size of the isopod populations in an ecosystem is determined mainly by the degree of tolerance of the species to the fluctuation of the ecological factors, a trait that influences the juvenile mortality rate and, consequently, the survival coefficient. Among the species that live in this area, *Protracheoniscus politus* has the highest tolerance to such fluctuations, which is reflected in the size of the populations and the wide dispersion of the individuals on the surface of the biotope.

8. In the studied ecosystems, in which the values of the ecological diversity and equitability are relatively high, for example certain spruce and beech forests, the communities of isopod species are formed by numerically reduced populations. The ecological conditions in these ecosystems are situated at the limits of the optimum of all species, and as a consequence, the juvenile mortality rate is high. For the isopod communities, these ecosystems can not be included in Thienemann's first biocoenotic principle.

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RESEARCH ON GROUNDWATER FAUNA IN NORTH-WESTERN ROMANIA

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SUMMARY. – This study presents the groundwater fauna in the interstitial and phreatic environments of two important Transylvanian basins (the area that is north-western Romania): Crişul Repede and Someşul. Differences between the quality of water in the two basins, the two biotopes and the sampling methods have allowed us to highlight the influence of surface water pollution on groundwater fauna in the Someşul Basin. Furthermore, we have assessed the heterogeneity of the habitats and high interstitial biodiversity in the Crişul Repede Basin.

The current objectives of groundwater research in Transylvania focus on biodiversity studies and on the impact of pollutants on the fauna, because groundwater fauna is largely ignored and represents an important part of the global biodiversity. It is greatly important because this fauna plays an active role in the correct functioning of the groundwater system [9].

To achieve these objectives, we have taken samples from different aquatic biotopes: basins in caves, karstic springs, wells, and interstitial water in the alluviums of caves or surface streams, of two of the most important Transylvanian hydrological basins: Crişul Repede and Someşul. This study presents only the results from wells (phreatic) and interstitial water.

Materials and methods. Fig. 1 shows the distribution of the sampling stations for groundwater fauna. For the sampling of wells, two methods were used: either by the filtration of 80 l of water (from most wells in the Someşul Basin) or by the use of a Cvetkov net [4] (in the Crişul Repede Basin). Depending on the sediments, interstitial water of the surface stream alluviums was also sampled using two methods: the Karaman-Chappuis method as described in [2] or the Bou-Rouch method [1]. The first consists of the digging of a hole in the sediments, into which water accumulates through capillary action. The Bou-Rouch method uses a diaphragm manual pump (such a device was made by a member of our team, G. Rajka). All the samples were filtered through a 200 µm sieve. In each and every case the depth and the quantity of filtered water were recorded and the data are presented in Table 1.

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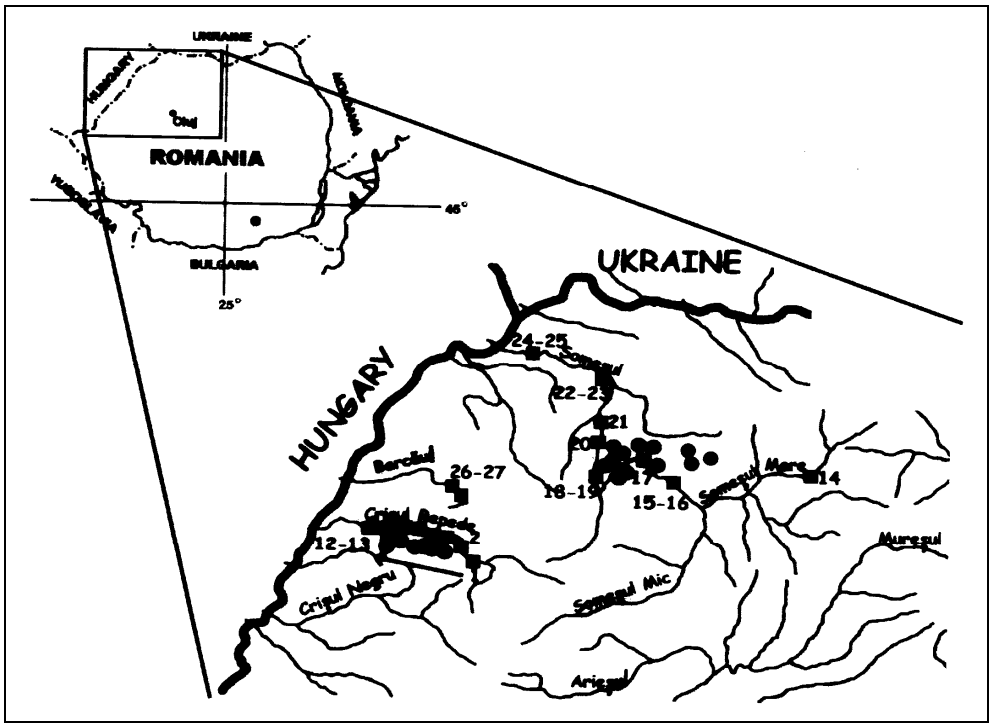


F i g. 1. *Sampling stations for groundwater fauna on the Crişul Repede River and the Someşul Basin*.
■ - Interstitial waters. ● - Wells (description of stations in Table 1).

Some of the groups have been identified at the genus level and others at the species level.

Water samples were also taken from other, complementary, locations, for chemical analysis. In some locations on the Someşul River, the structure and clogging of the sediments rendered impossible any of the sampling methods. At the same time, in other locations, no fauna was found, which was especially true on the Barcău River, one of the Crişul Repede tributaries. In this case the samples were not considered in the analysis.

We will present the results of two series of sampling campaigns undertaken during summer - autumn 2000, and also in spring - autumn 2001.

Certain physical-chemical parameters of the water (temperature, pH, conductivity and chemical oxygen demand – CDO-Mn) were measured during the field sampling or in the laboratory. Temperature, pH and conductivity were measured with electronic portable instruments: Piccolo plus and Conmet 1, respectively.

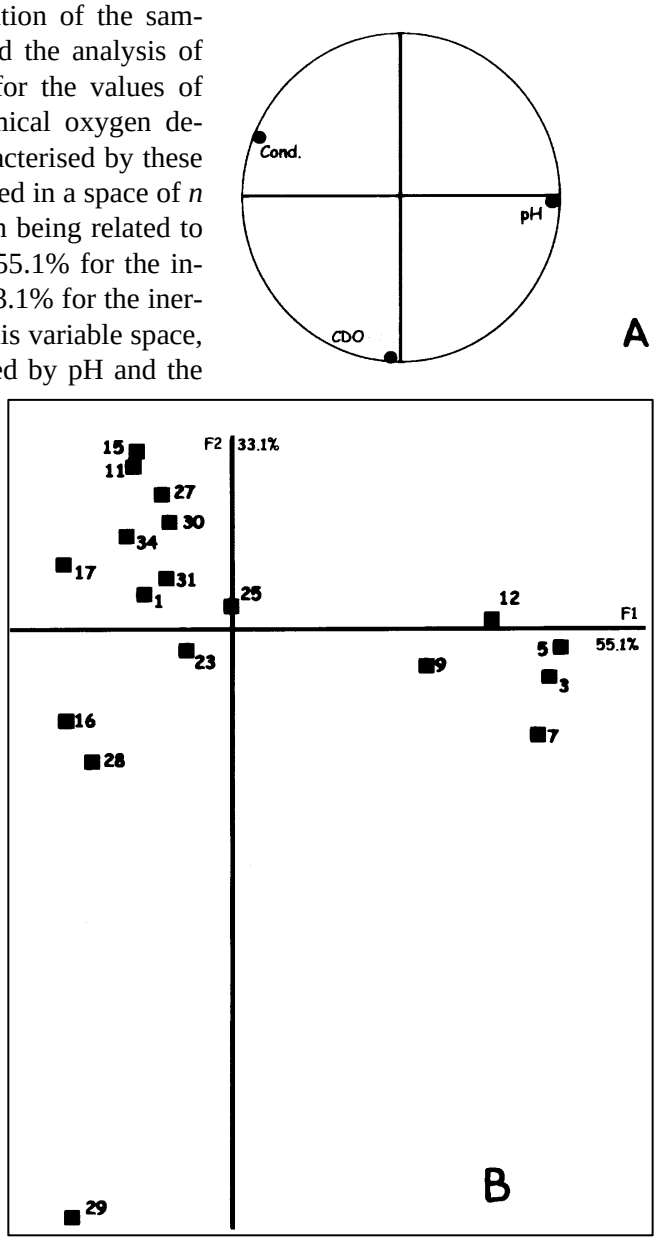
Table 1

Physical features of the groundwater fauna samples on the Crişul Repede, Someşul and Barcăul rivers

No.	Station	Physical features	
		Bou-Rouch pumping	Karaman-Chappuis sampling
		CRIŞUL REPEDE	
1	Bologa	10 l filtered water 1 m deep; sediment: sand and gravel	
2	Negreni	10 l fileterd water 0.7 m deep; sediment: sand and gravel	
3	Bulz	10 lo filtered water 0.7 m deep; sediment: sand and gravel	
4	Lorău		20 l filtered water; sediment: fine gravel
5	Lorău		20 l filtered water; sediment: fine gravel
6	Şuncuiuş	44 l filtered water 1 m deep; sediment: sand and gravel	
7	Şuncuiuş		20 l filtered water sediment: very fine sand
8	Vadu-Crişului		20 l filtered water sediment: sand and gravel
9	Vadu-Crişului		20 l filtered water sediment: sand and gravel
10	Auşeu	5 l filtered water 0.7 m deep; sediment: sand and gravel	
11	Aştileu	10 l filtered water 0.5 m deep; sediment: sand and gravel	
12	Aleşd	10 l filtered water 1 m deep; sediment: gravel	
13	Aleşd		20 l filtered water; sediment: gravel
		SOMEŞUL	
14	Beclean	10 l filtered water 0.7 m deep; sediment: gravel	
15	Gâlgăău	10 l filtered water 0.3 m deep; sediment: sand	
16	Gâlgău		20 l filtered water sediment: fine sand
17	Ileanda	10 l filtered water 1 m deep; sediment: sand and gravel	
18	Jibou	10 l filtered water 0.5 m deep; sediment: sand and gravel	
19	Jibou		10 l filtered water sediment: sand with grey cover
20	Ulmeni	10 l filtered water 1 m deep; sediment: sand and gravel	
21	Tămaia	10 l filtered water 1 m deep; sediment: sand and gravel	
22	Cicârlău	10 l filtered water 0.5 m deep; sediment: sand and gravel	
23	Cicârlău		10 l filtered water sediment: sand and gravel
24	Satu Mare	1 l filtered water 0.3 m deep; sediment: sand	
25	Satu Mare		2 l filtered water sediment: very fine sand
		BARCĂUL	
26	Nuşfalău 1	10 l filtered water 0.5 m deep; sediment: sand and gravel	
27	Nuşfalău 2		10 l filtered water sediment: sand and gravel

Results. Results of physical-chemical measurements are shown in Table 2. As one can notice, there are important differences between the quality of surface and interstitial waters when all the parameters are taken into account.

For a better separation of the sampling stations we have used the analysis of the principal components for the values of pH, conductivity and chemical oxygen demand. Each sample is characterised by these 3 variables and can be located in a space of *n* dimensions, each dimension being related to a variable. The values are 55.1% for the inertia for the first axis and 33.1% for the inertia for the second axis. In this variable space, the first axis is characterised by pH and the second axis by chemical demand of oxygen (Fig. 2A). In the space of populations (Fig. 2B), the samples from the Crişul Repede River are well separated from those from the Someşul River, excluding the first up-stream (Bologa) and the last down-stream (Toboliu) stations. Thus, most of the Crişul Repede stations are well defined and distinguished by their pH. At the same time, all the other stations are defined by the conductivity, with one exception - the station Cicârlău on the Someşul River, which has a completely separate position due to the high value of its chemical oxygen demand.



F i g . 2 . *Principal component analysis.* A - Correlation circle. B - Representation of physical-chemical features of the water samples (see Table 2 for the numbers).

Table 2

Physical-chemical characteristics of the sampled groundwater
(B-R - Bou-Rouch pumping. K-C - Karaman-Chappuis sampling)

No.	Sample	Temperature (°C)	pH	Conductivity (µS)	CDO-Mn (mg/l)
		CRIŞUL REPEDE			
1	Bologa - stream	-	7.0	586	32.1
2	Bologa - B-R	10.3	8.4	533	-
3	Negreni - stream	-	8.9	250	23.2
4	Negreni - B-R	12.3	7.9	264	-
5	Bulz - stream	-	9.2	310	22.4
6	Bulz - B-R	11.1	8.6	266	-
7	Auşeu - stream	-	8.4	173	26.7
8	Auşeu - B-R	12.8	8.3	265	-
9	Aştileu - stream	-	7.7	256	22.4
10	Aştileu - B-R	13.6	8.4	266	-
11	Toboliu - stream	-	7.9	796	26.1
		SOMEŞUL			
12	Năsăud - stream	15.2	8.7	326	20.6
13	Beclean - stream	-	6.1	390	23.0
14	Beclean - B-R	15.5	7.5	830	-
15	Gâlgău - stream	8.3	6.4	562	12.3
16	Gâlgău - K-C	9.3	6.4	562	46.6
17	Gâlgău - B-R	9.5	6.4	631	30.8
18	Ileanda - stream	-	8.1	553	26.9
19	Ileanda - B-R	14.8	-	-	-
20	Jibou - stream	-	-	-	9.8
21	Jibou - K-C	14.5	6.6	-	-
22	Jibou - B-R	15.5	6.5	-	-
23	Ulmeni - stream	-	7.2	543	35.3
24	Ulmeni - B-R	14.8	7.6	565	
25	Tămaia - stream	-	7.5	510	28.3
26	Tămaia - B-R	15.3	7.3	779	-
27	Cicârlău - stream	2.6	6.5	525	15.5
28	Cicârlău - K-C	9.5	6.6	540	50.6
29	Cicârlău - B-R	10.1	6.6	401	101.1
30	Satu Mare - stream	7.9	6.5	505	18.6
31	Satu Mare - B-R	6.1	6.4	476	22.9
		BARCĂUL			
32	Nuşfalău 1 - B-R	12.6	6.6	-	-
33	Nuşfalău 2 - K-C	14.5	6.5		
34	Roşiori - stream	-	6.9	626	25.9

Concerning the groundwater fauna, Table 3 presents the distribution in the abundance of different groups in wells and Table 4 shows their abundance in the surface water alluviums. The distributions were calculated for each station (column) and also for each group (line).

We will first analyse communities of wells (phreatic) and the interstitial communities of the two basins. We will then analyse the fauna from the two biotopes and the sampling methods.

Crişul Repede

Wells (phreatic):

– from the 11 identified fauna groups more than half (52.1%) of the subterranean representatives belong to Cyclopoida;

– most of the Cyclopoida are stygoxenic (*Megacyclops viridis*) or stygophilous (*Diacyclops crassicaudis*, *D. bicuspidatus*, *Paracyclops fimbriatus* and *Eucyclops serrulatus*) species;

– other representatives belong to Isopoda, Ostracoda, Oligochaeta, Amphipoda (*Niphargus* sp.) and Hydracari in decreasing order of their distribution; the Collembola, Gastropoda, Cladocera, insect larvae and Nematoda are poorly represented (Table 3; Fig. 3).

Interstitial:

– individuals from 14 fauna groups have been identified: Nematoda, Oligochaeta, Lamellibranchiata, Cladocera, Cyclopoida, Harpacticoida, Ostracoda, Tardigrada, Amphipoda (*Niphargus* sp.), Isopoda, Bathynellacea, Hydracari, Collembola and insect larvae (Table 4; Figs. 4 and 5);

– Oligochaeta, Copepoda and Nematoda are dominant in these communities, with the following few exceptions:

– at small depths (0.2 to 0.3 m) the biological communities are dominated by Nematoda and Oligochaeta, while for greater depths (0.5–1.0 m), the stygobiontic *Parastenocaris* represents more than 50%;

– along the river, the Cyclopoida that dominate the up-stream communities are being replaced down-stream by Harpacticoida, Nematoda and Oligochaeta; beyond the city of Aleşd, the copepods are almost completely absent and the Nematoda and Oligochaeta become associated (in more than 75% of the stations) with an unexpected increase in the number of water mites (Figs. 4 and 5).

Someşul

Wells (phreatic):

– more than 95% are the Cyclopoida copepods, while other groups (Ostracoda, Harpacticoida, Hydracari, Amphipoda, Collembola, Isopoda, insect larvae, Nematoda, Oligochaeta, Gastropoda) are small in number and are found only in a few samples (Table 3; Fig. 3);

Table 3

Fauna in wells (phreatic) of the Crișul Repede and the Someșul basins
(A - Abundance and F - Frequency of taxonomic groups* - Cvetkov net samples)

Station	*Nemat.*	*Olig.*	*Gastr.*	*Clado.*	*Cyclo.*	*Harpa.*	*Ostra.*	*Amphi.*	*Isopo.*	*Acari*	*Collem.*	*Insect larvae*	*A%*
CRIȘUL REPEDE													
*Bulz 16	-	1	10	-	187	-	179	27	45	-	-	1	*13.60*
*Bulz 116	-	13	-	22	54	-	-	1	-	-	1	-	*2.75*
*Lorău 176	3	64	4	-	172	-	1	17	59	-	-	-	*9.67*
*Bratca 425	-	-	2	-	52	-	8	13	235	13	1	2	*9.85*
*Bălnaca	1	-	-	-	123	-	1	2	3	-	10	-	*4.23*
*Vadu Criș	-	1	-	-	24	-	-	2	18	-	-	-	*1.36*
*Butan 85	-	81	10	-	79	-	-	-	4	8	14	8	*6.16*
*Butan 56	-	96	-	-	21	-	-	1	7	1	5	2	*4.02*
*Aștilcu 27	-	1	1	-	25	-	6	174	19	13	-	-	*7.22*
*Aștilcu 140	-	27	-	-	987	-	119	11	163	52	-	2	*41.13*
A%	*0.12*	*8.58*	*0.82*	*0.66*	*52.10*	*0*	*9.49*	*7.49*	*16.71*	*2.63*	*0.94*	*0.45*	
F%	*20*	*80*	*50*	*10*	*1000*	*0*	*60*	*90*	*90*	*50*	*50*	*50*	
SOMEȘUL													
*Gilău 2	-	-	1	-	44	-	182	11	-	-	1	1	
*Gilău 36	1	-	-	-	20	-	10	19	-	-	-	-	
*Gilău 125	-	-	-	-	252	-	117	27	3	1	1	1	
A%	*0.14*	*0*	*0.14*	*0*	*45.66*	*0*	*44.65*	*8.24*	*0.43*	*0.14*	*0.29*	*0.29*	
Lelești 24	-	-	-	-	16	-	9	5	-	1	-	-	*0,66*
Lelești 29	-	-	-	-	395	1	16		-	-	-	-	*8,90*
Lelești	-	-	-	-	127	-	-	1	1	1	2	-	*2,85*
Ciccu	-	-	1	-	1	-	-	-	-	-	-	3	*0.11*
Răstoci	-	-	-	-	1	-	-	-	-	1	-	-	*0.04*

Table 3

Fauna in wells (phreatic) of the Crișul Repede and the Someșul basins
(A - Abundance and F - Frequency of taxonomic groups* - Cvetkov net samples)

Station	*Nemat.*	*Olig.*	*Gastr.*	*Clado.*	*Cyclo.*	*Harpa.*	*Ostra.*	*Amphi.*	*Isopo.*	*Acari*	*Collem.*	*Insect larvae*	*A%*
CRIȘUL REPEDE													
*Bulz 16	-	1	10	-	187	-	179	27	45	-	-	1	*13.60*
*Bulz 116	-	13	-	22	54	-	-	1	-	-	1	-	*2.75*
*Lorău 176	3	64	4	-	172	-	1	17	59	-	-	-	*9.67*
*Bratca 425	-	-	2	-	52	-	8	13	235	13	1	2	*9.85*
*Bălnaca	1	-	-	-	123	-	1	2	3	-	10	-	*4.23*
*Vadu Criș	-	1	-	-	24	-	-	2	18	-	-	-	*1.36*
*Butan 85	-	81	10	-	79	-	-	-	4	8	14	8	*6.16*
*Butan 56	-	96	-	-	21	-	-	1	7	1	5	2	*4.02*
*Aștileu 27	-	1	1	-	25	-	6	174	19	13	-	-	*7.22*
*Aștileu 140	-	27	-	-	987	-	119	11	163	52	-	2	*41.13*
A%	*0.12*	*8.58*	*0.82*	*0.66*	*52.10*	*0*	*9.49*	*7.49*	*16.71*	*2.63*	*0.94*	*0.45*	
F%	*20*	*80*	*50*	*10*	*1000*	*0*	*60*	*90*	*90*	*50*	*50*	*50*	
SOMEȘUL													
*Gilău 2	-	-	1	-	44	-	182	11	-	-	1	1	
*Gilău 36	1	-	-	-	20	-	10	19	-	-	-	-	
*Gilău 125	-	-	-	-	252	-	117	27	3	1	1	1	
A%	*0.14*	*0*	*0.14*	*0*	*45.66*	*0*	*44.65*	*8.24*	*0.43*	*0.14*	*0.29*	*0.29*	
Lelești 24	-	-	-	-	16	-	9	5	-	1	-	-	*0,66*
Lelești 29	-	-	-	-	395	1	16		-	-	-	-	*8,90*
Lelești	-	-	-	-	127	-	-	1	1	1	2	-	*2,85*
Ciceu	-	-	1	-	1	-	-	-	-	-	-	3	*0.11*
Răstoci	-	-	-	-	1	-	-	-	-	1	-	-	*0.04*

Table 4

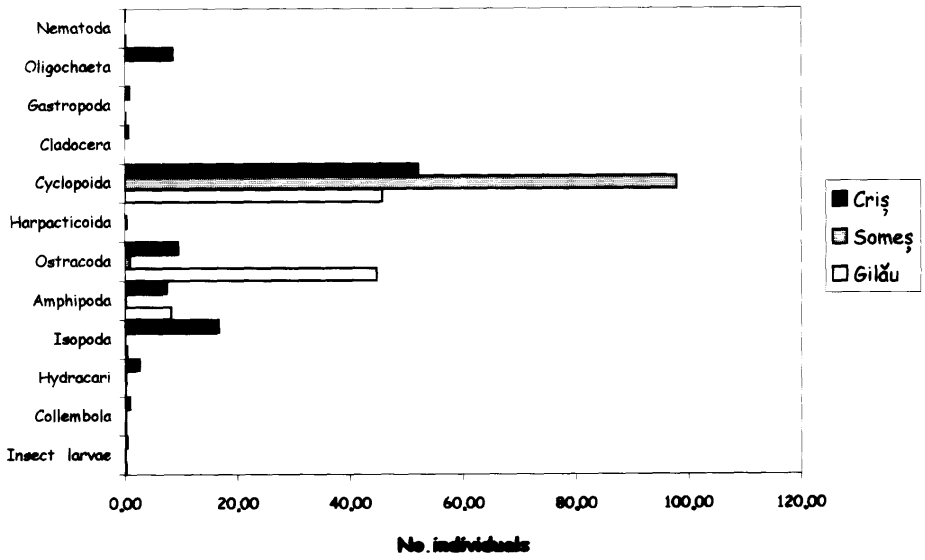
Fauna of interstitial waters (no. of individuals/10 l water) sampled with Bou-Rouch and Karaman-Chappuis methods in the Crișul Repede (with the tributary Barcăul) and the Someșul basins

Station	*Nemat.*	*Olig.*	*Lamelli.*	*Clado.*	*Cyclo.*	*Harpa.*	*Ostra.*	*Tardig.*	*Amphi.*	*Isopo.*	*Bathyn.*	*Acari*	*Collem.*	*Insect larvae*	*No. ind.*
CRIȘUL REPEDE															
Bou-Rouch															
Bologa	4	13	-	-	90	4	1	-	-	-	-	1	-	3	116
Negreni	8	30	-	-	35	26	4	-	-	16	5	1	-	4	129
Bulz	1	1	-	-	-	9	-	-	-	-	-	3	-	6	20
Șuncuiuș	8	6	-	1	22	122	-	-	-	-	-	1	-	13	173
Ausșeu	62	102	-	-	10	382	-	-	-	-	-	-	-	-	556
Aștileu	3	1	-	-	5	11	-	-	-	-	-	2	2	6	30
Aleșd	12	-	-	-	-	-	-	-	-	-	-	1	-	1	14
Karaman-Chappuis															
Lorău 1	17	6	-	7	3	7	1	-	-	-	-	5	2	4	52
Lorău 2	40	18	-	5	8	12	1	-	-	-	-	2	2	10	98
Șuncuiuș	232	55	-	3	8	88	-	5	-	-	5	2	6	19	423
Vadu Criș 1	4	5	-	-	-	-	-	-	-	-	-	-	-	2	11
Vadu Criș 2	10	34	-	-	2	-	-	-	1	-	-	-	-	6	53
Aleșd	307	348	14	-	4	14	-	2	-	-	-	101	-	57	847
A %	*28.07*	*24.54*	*0.56*	*0.63*	*7.41*	*26.76*	*0.28*	*0.28*	*0.04*	*0.63*	*0.40*	*4.72*	*0.48*	*5.19*	
P %	*100*	*92.3*	*7.7*	*30.8*	*76.9*	*76.9*	*30.8*	*15.4*	*7.7*	*7.7*	*15.4*	*76.9*	*30.8*	*92.3*	

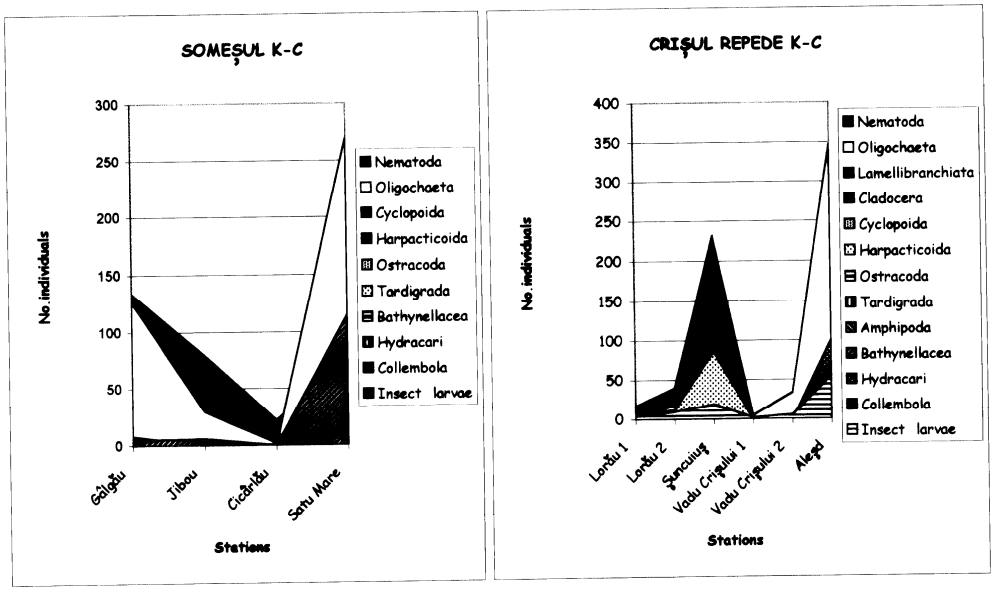
Table 4 (continued)

Fauna of interstitial waters (no. of individuals/10 l water) sampled with Bou-Rouch and Karaman-Chappuis methods in the Crișul Repede (with the tributary Barcăul) and the Someșul basins

Station	*Nemat.*	*Olig.*	*Lamelli.*	*Clado.*	*Cyclo.*	*Harpa.*	*Ostra.*	*Tardig.*	*Amphi.*	*Isopo.*	*Bathyn.*	*Acari*	*Collem.*	*Insect larvae*	*No. ind.*
SOMEȘUL															
Bou-Rouch															
Beclean	179	-	-	-	-	-	2	-	-	-	-	2	-	4	187
Gilău	57	165	-	-	25	-	15	1	-	-	-	1	-	5	269
Ileanda	2	1	-	-	-	-	-	-	-	-	-	-	-	-	3
Jibou	25	23	-	-	-	-	1	-	-	-	-	-	1	5	55
Ulmeni	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
Tămaia	-	-	-	-	1	-	1	-	-	-	-	-	-	-	2
Cicârlău	28	5	-	-	1	-	1	-	-	-	-	-		16	51
Satu Mare	10	140	-	-	-	-	-	-	-	-	-	-	-	15	165
Karaman-Chappuis															
Gâlgău	134	127	-	-	8	-	-	-	-	-	6	-	-	5	280
Jibou	80	30	-	-	-	1	-	-	-	-	-	1	1	6	119
Cicârlău	-	2	-	-	-	-	-	1	-	-	-	-	-	-	25
Satu Mare	80	270	-	-	-	-	10	15	-	-	-	-	-	115	490
A %	*37.46*	*46.33*			*2.13*	*0.06*	*1.82*	*1.03*			*0.36*	*0.24*	*0.18*	*10.38*	
F %	*83.3*	*75.0*			*33.3*	*8.3*	*50.0*	*25.0*			*8.3*	*25.0*	*25.0*	*66.7*	
BARCĂUL															
Bou-Rouch															
Nușfalău 1	13	9	-	-	-	5	-	-	-	-	-	-	-	4	31
Karaman-Chappuis															
Nușfalău 2	11	134	-	-	19	44	37	-	-	-	-	8	-	43	296
A %	*7.34*	*43.73*	*0*	*0*	*5.81*	*14.98*	*11.31*	*0*	*0*	*0*	*0*	*2.45*	*0*	*14.37*	



F i g. 3. *Abundance-distribution of fauna groups in wells of the Crişul Repede and the Someşul basins.*



F i g. 4. *Interstitial fauna groups in Karaman-Chappuis samplings of the Crişul Repede and the Someşul rivers.*

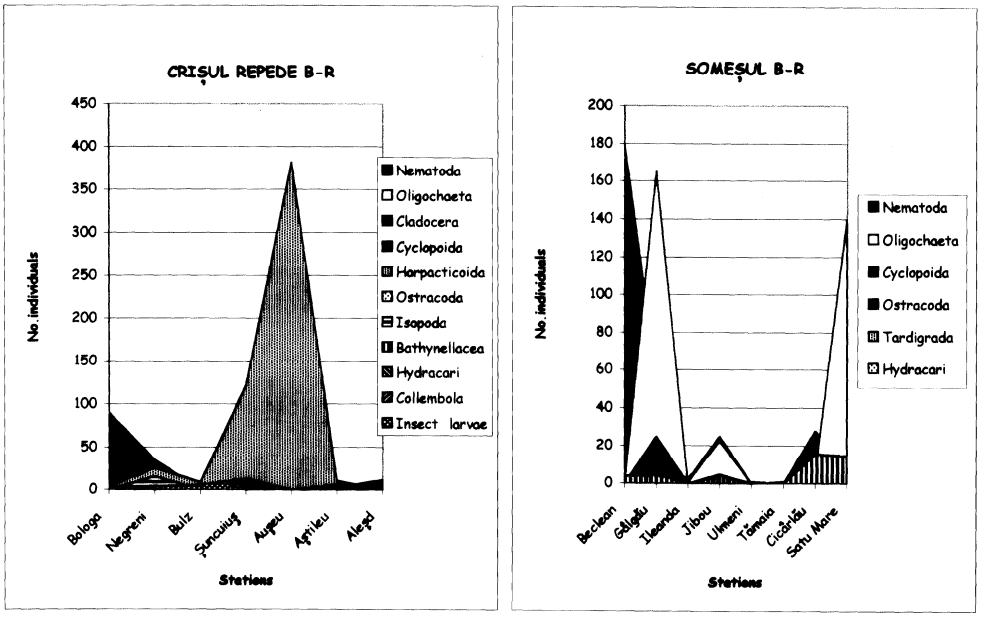
– belonging to the Cyclopoida group, the dominant species is *Megacyclops viridis*; in addition, there have also been identified *Diacyclops bicuspidatus*, stygoxenic and *D. languidoides*, a stygophilous species; *D. bicuspidatus* was almost always found to be associated with *M. viridis*, with the exception in one sample where it was found alongside the other species of the genus;

– samples from Gilău are different from those from the Someşan Plateau: two groups, Cyclopoida and Ostracoda share the dominance; the *Niphargus* are in smaller number (over 8%) and there are also other groups in very small proportions: Isopoda, Collembola, insect larvae, Hydracari, Nematoda and Gastropoda.

Interstitial:

– 10 fauna groups can be found in this environment: Nematoda, Oligochaeta, Cyclopoida, Ostracoda, insect larvae; a very small number of stations have also Collembola, Hydracari, Harpacticoida, Tardigrada, Bathynellacea (Table 4; Figs. 4 and 5);

– more than 80% of the individuals are stygoxenic Oligochaeta and Nematoda, irrespective of the depth and all along the river; the exceptions being found in the down-stream stations, which are rich in Oligochaeta, larvae of Chironomidae and Nematoda.



F i g. 5. *Interstitial fauna groups in Bou-Rouch samplings of the Crişul Repede and the Someşul rivers.*

Barcăul

Interstitial:

– it must be mentioned that this station is near the source, up-stream from most of the sources of pollution; the sampling stations down-stream completely lack this fauna;

– the results of the Bou-Rouch pumping method showed poor biodiversity, finding only a few individuals, belonging to 4 groups: Nematoda, Oligochaeta, Harpacticoida and insect larvae; the results of the superficial Karaman-Chappuis sampling showed a richer and more diverse fauna - in this case, the Oligochaeta are dominant, followed by Harpacticoida, insect larvae, Ostracoda, Cyclopoida, Nematoda and Hydracari (Table 4).

As mentioned before, there are some differences between stations depending upon the sampling method. With regard to the wells (Fig. 3), the differences between the locality of Gilău and the Someşan Plateau are mainly between the dominance of two and one group, respectively. Fig. 6 compares the two methods of sampling interstitial fauna. On the Crişul Repede River the two methods (Bou-Rouch and Karaman-Chappuis) reveal almost completely different communities. On the Someşul River, on the contrary, the communities are dominated, irrespective of method and depth, by the same groups: Oligochaeta, Nematoda and Diptera larvae.

In order to emphasise the influence of surface water pollution on groundwater fauna we have compared (Fig. 7) the fauna from the wells (which should give the right measure of the phreatic biodiversity) with the interstitial fauna. It appears that the fauna in the two environments is very similar and that there are important differences between these environments inside the same basin. In the Someşul Basin, the wells are characterised by strong communities of Cyclopoida, while Nematoda and Oligochaeta dominate the interstitial water. Crişul Repede has other groups, which represent an important part of the interstitial communities; these are Harpacticoida, Cyclopoida, insect larvae (mostly Diptera but also those belonging to Ephemeroptera, Trichoptera and Plecoptera) and water mites. Some species of the Crişul Repede are stygobiontic, and apparently, as not all species were identified, the interstitial water of the Someşul River has no stygobiontic species, except some Bathynellacea which were found in one station.

Discussion. The Someşul River is well known in Romania as being very polluted. Particular cases, exceeding the admissible limits for class III (β mesosaprobious water), have been recorded on the following sections: Salatiu (P) on the Someşul Mic River; Răstoci, Ulmeni, Cicârlău, Ambud and Oar on the Someş River. The afore-mentioned cases of pollution are mainly caused by the chemical industry (Terapia Cluj, Someş Dej), metalworks (S.C. Phoenix S.A. Baia Mare), extractive industry (E.M. Baia Sprie, E.M. Herja, E.M. Cavnic) and animal farms (Avicola Satu Mare, Agrocomsuin Bonțida, Comsuin Moftin) [10].

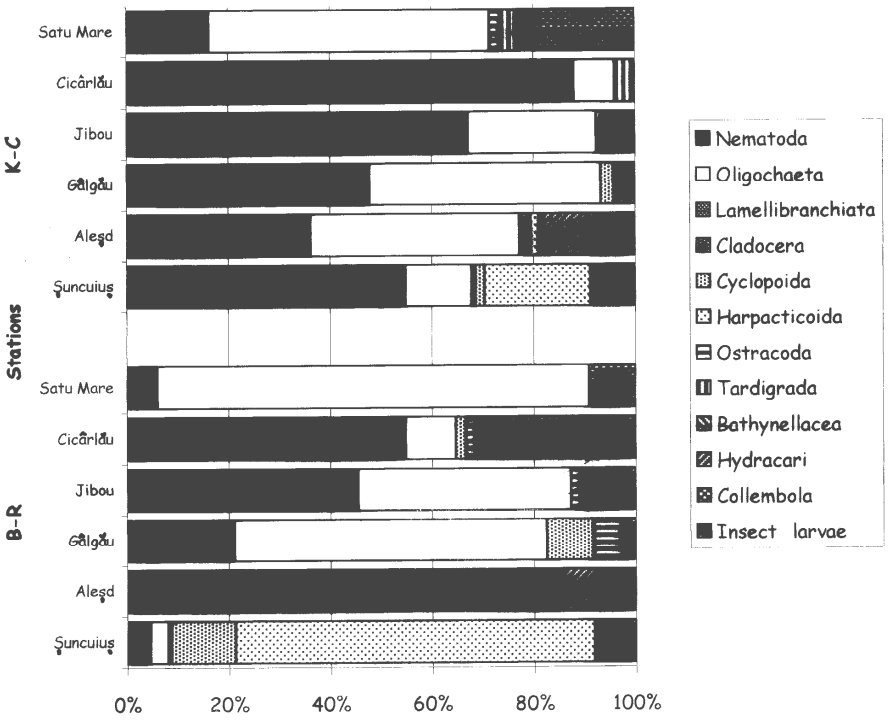
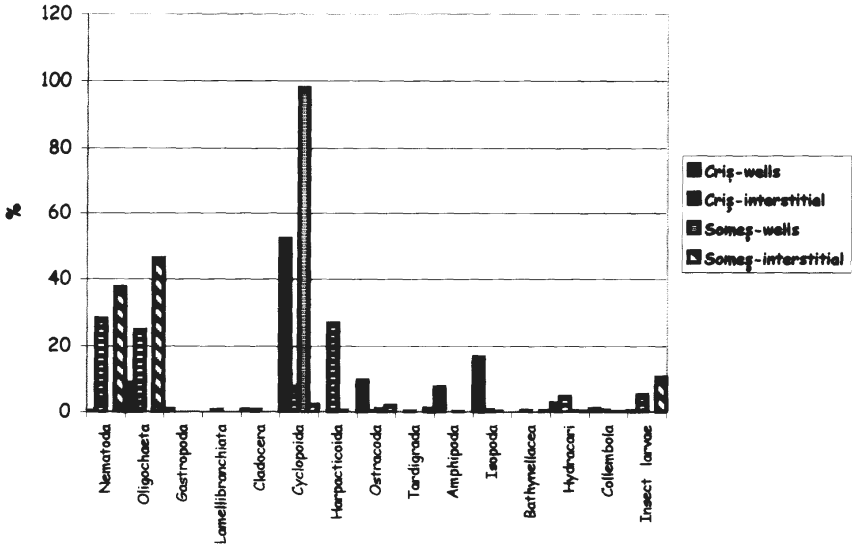


Fig. 6. *Comparison between the interstitial fauna sampled by Karaman-Chappuis and Bou-Rouch methods (reflecting also the distribution of groups in depth).*



F i g. 7. *Comparison between the abundance of fauna groups in interstitial waters and wells in the Crişul Repede and the Someşul basins.*

In the Criş hydrographical basin, pollution over the admissible limits for class III (β mesosaprobious water) has been recorded on the following sections: downstream from Suplacu (oil products) and Parhida (oil products) on the Barcău River. In the Barcău River basin, crude oil extraction and industrial works have been found, originating from Petrom's works in Suplacu de Barcău [10].

The Report from the Romanian Ministry for Water and Protection of the Environment [10] indicates further regions where the groundwater is highly polluted, with concentrations of pollutants exceeding the limits specified by the standard for this indicator, STAS 1342-91; amongst these regions is the Someş lower plain. During the year 2000, the highest pollution was recorded, amongst other basins, in the Someş Basin on a 26-km stretch (Lăpuş–7 km, Săsar–19 km), where the benthonic fauna was absent over the whole sampling period.

Our chemical analysis agrees with the Ministerial Report mentioned above and completely distinguishes the polluted waters (Someşul and Barcăul) from the clean Crişul Repede. Moreover, our results reflect the differences in the water quality at different depths compared with the surface water. Thus, the interstitial samples show that the pollutants are either not present or their concentration is sometimes low in the surface water. It is known that groundwater can become concentrated with pollutants, and therefore reflects not only recent episodes of pollution but also episodes in the past. This problem becomes extremely critical, because these substances are more concentrated at greater depths and thus will enter the phreatic water, which is used by large human communities, as in our area of study. The two rivers are also different from this point of view. Crişul Repede River has more individuals between 0.5 and 0.7 m depth and the biggest biodiversity between depths of 0.3 and 0.2 m, which is the layer where the mixture between stygophilous and stygobiontic fauna is most important. Someşul River is almost completely depopulated at greater depths, whereas at 0.7 m the Nematoda are dominant in these communities, and near the surface the Oligochaeta and Diptera larvae have more individuals. Comparisons between and within each of the two basins revealed differences between:

– up-stream and down-stream stations on each river; the differences are much more pronounced on Crişul Repede River, thus giving the measure of the diversity of interstitial habitats, which are almost absent on Someş due to the poor quality of the water;

– the two basins in the general biodiversity, number of individuals and presence of stygobiontic species;

– interstitial waters and wells inside the same basin;

– the two sampling methods of the groundwater fauna (Bou-Rouch and Karaman-Chappuis).

The decrease in the number of fauna groups, individuals and stygobiontic forms at the down-stream stations on Someşul and Barcăul rivers proves the influence of the polluted surface water on the interstitial water. Biological indicators of pollution dominate these communities, such as Oligochaeta and Nematoda. On the Barcău River, which is very affected by the animal farms and the oil platforms, the situation is similar to the Someş River: very few groups are represented and almost half of the individuals are Oligochaeta. In such cases we have extreme environments with poor diversity, with few or even one species developing important populations. On the contrary, the biodiversity on the Crişul Repede River is incomparably greater in both the wells and the interstitial waters, and there are also many stygobiontic species.

Differences in the composition of these communities, measured by sampling in interstitial waters, especially on Crişul Repede River, raise the necessity of using both methods to highlight the biodiversity in the subterranean superficial waters. The quality of the water is also the main factor in maintaining the different habitats at different levels or at different locations, due to the fact that pollution will affect all the levels of the ecotone represented by interstitial water.

Sampling in wells also gives different results because the Cvetkov net ensures the sampling of the animals living in the bottom sediments, like Ostracoda. This environment displays differences between the two basins similar with those between interstitial waters, as a higher biodiversity (for example, half of the wells in Someş have only 2 species of Cyclopoida). This is one of the reasons for which the pollution on Someşan Plateau extended also into the phreatic water, the probable causes being the agricultural practices and the very thin soil. The differences between Gilău and the Someşan Plateau, belonging to the same basin, can be given by the different sampling method or by the quality of the water.

Our studies in two of the most important Transylvanian hydrological basins emphasised the importance of the protection of the surface water, in order to maintain the quality of the groundwater. Many authors have already demonstrated that groundwater contamination induces a decline in the diversity and density of stygobionts [3, 7, 8]. Gibert *et al.* [5] have recognised, through research on groundwater/surface water ecotones, that processes occurring in surface and groundwater environments are influenced by the riparian and hyporheic zones. These ecotones are important landscape features because of their physical and biological characteristics, and their unique spatial and temporal characteristics [12]. In the Someşul Basin, the groundwater, at any level, is seriously affected in its biodiversity and our preliminary studies near the source of the river point to the seriousness of the problem [11]. At present, we can not estimate if this area can recover if protection measures are applied. The impact of pollution is severe, yet the Crişul Repede is still a depository of rich fauna [6]. Therefore, given the gravity of the consequences, it is imperative that the fauna of the Crişul Repede must be preserved post-haste.

Conclusions. Our study is a comparison between groundwater biodiversity of two important Transylvanian basins, Crişul Repede and Someşul.

1. The clean Crişul Repede River is still the depository of many interstitial organisms, while the polluted Someşul has poor interstitial communities dominated by Oligochaeta and Nematoda.

2. The results concerning the interstitial fauna are consistent with the fact that surface waters are polluted in the Someşul Basin.

3. Stygobiontic species were found only in the interstitial of the Crişul Repede River.

4. The importance of maintaining unpolluted interstitial and phreatic habitats is given by the heterogeneity of their groundwater communities.

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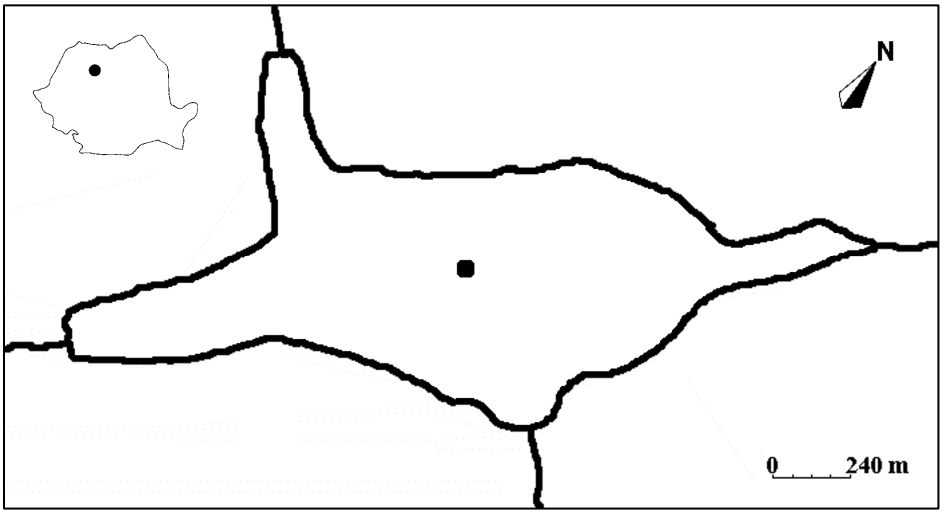
STRUCTURE AND DYNAMICS OF THE ZOOPLANKTON COMMUNITY IN THE ŞTIUCII LAKE (NORTH-WESTERN ROMANIA)

TRAIAN BRAD*

SUMMARY – In a pelagic station of the Ştiucii Lake situated in North-Western Romania two species of copepods (*Cyclops vicinus* Uljanin, 1875 and *Mesocyclops* (*Thermocyclops*) *crassus* Rehberg, 1880) and four species of cladocerans (*Daphnia galeata* Sars, 1864; *Bosmina longirostris* O.F.M., 1785; *Ceriodaphnia pulchella* Sars, 1862; *Chydorus sphaericus* O.F.M., 1785) were identified. Their density and population structure were seasonally changing. Density of these organisms increased at higher temperatures of the lake water, respectively from winter to summer, and then decreased in autumn. In the seasonal succession of the zooplankton community, *Mesocyclops* (*Thermocyclops*) *crassus* replaced *Cyclops vicinus* in summer. *Ceriodaphnia pulchella* (Cladocera) appeared in summer and then disappeared in autumn. In the population structure of copepods a dominance of the males was observed at every sampling. The maximum fertility of the cladoceran species was registered in spring (*Bosmina longirostris*) and in summer (*Daphnia galeata*).

The Ştiucii lake (Fig. 1) is situated in Transylvania (North-Western Romania). It is located at an altitude of 282 m and has a SSW-NNE general orientation. The lake is bounded by hills with heights between 470 and 520 m and has an oblong shape with four inlets corresponding to its tributaries: Vănăului Valley and Sănăşele Valley from the left, and Arnitei Valley and Săcălaia Valley – from the right.



F i g . 1. *Plan of the Ştiucii Lake with the location of the sampling station.*

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On the basis of the dominant species of algae (*Ceratium hirundinella*, *Peridinium* sp., *Coelastrum* sp., *Scenedesmus* sp., *Pediastrum* sp.), it was inferred that the Ştiucii Lake is presently at the beginning of a eutrophic phase [4, 6].

In 1966, the Ştiucii Lake became a Nature Reserve of the Romanian Academy [8] according to the Decree 244, state reconfirmed in 1974 through the Decree 686.

The morphometric characteristics of the lake are: surface area = 68.7 ha, length = 1.72 km, maximum width = 826 m, medium width = 399 m, volume = 1,883,960 m^3.

The depth was measured by sounding the lake bottom with a plumb. It was found, based on 173 measurements performed over transversal alignments, that the maximum depth was 7 m, in contradiction with the results of Săndulache and Buta [7], who reported a maximum depth of over 12 m. The difference may be due to siltation.

Geologically, the lake covers a diapir area with salt outcrops, the evaporite deposit being isolated from the lake water by a thick layer of mud. The solution and incision processes are momentarily stopped. Due to the former process of salt solution, the Ştiucii Lake is one of the deepest natural lakes in the Transylvanian Depression.

Materials and methods. The samplings were carried out during four seasons, from February 2000, when the lake was covered with a 40-cm ice layer, until November 2000.

The physicochemical properties (temperature in ^oC, pH, dissolved oxygen in mg/l, photosynthetically available radiation in µmol/s/m^2, salinity in mg/l, conductivity in µS/cm, Secchi disk transparency in m) of the lake water (Table 1) were measured at 1-m intervals at every sampling.

The samples were taken from a single pelagic station at 1-m intervals down to the depth of 6 m. For zooplankton sampling a Schindler-Patalas trap with a capacity of 10 l was used. A volume (V) of 30 l for each sample was filtered through a planktonic net with a mesh size of 0.065 mm, the dross having been then transferred to plastic jars. The dross was treated with a 4% sugar solution to avoid the loosing of eggs of the egg-carrying females due to the shock formalin (4%) preservation. In the laboratory, the dross from the plastic jars was washed and brought to 30 ml (V_1), of which a third part (V_2) was analysed in a counting chamber at the stereomicroscope.

Density of the zooplanktonic organisms from the Ştiucii Lake community was calculated using the following formula:

$$N = \frac{n}{fa \times V},$$

where ***N*** is the density (number of specimens per m^3 of water); *n*, the number of specimens counted in the subsample; *fa*, the analysed fraction (the proportion between

V_2 and V_1, in our case 10/30 = 0.33333).

When low densities of organisms were encountered, we examined the entire sample.

Results and discussion. The measurement of physicochemical properties of the lake (Table 1) showed a variation with depth and season.

Table 1

Seasonal variation of the physicochemical characteristics of the Ştiucii Lake

Date/Depth (m)	0	1	2	3	4	5	6
Temperature (^oC)							
29 Feb.	0.6	3.1	3.5	3.7	3.7	3.5	3.6
30 Mar.	8.6	8.2	4.5	4.0	3.4	3.6	5.0
14 Apr.	12.1	10.3	9.4	8.5	7.5	7.1	6.2
15 Aug.	29.9	28.5	28.1	28.1	24.2	19.4	18.9
16 Nov.	9.4	9.3	9.1	9.0	9.0	9.0	9.0
pH							
30 Mar.	8.0	8.0	7.8	7.7	7.7	7.7	7.6
14 Apr.	8.3	8.2	8.2	8.1	7.9	7.8	7.6
15 Aug.	7.9	7.8	7.7	7.4	7.1	6.7	6.6
Oxygen (mg/l)							
29 Feb.	13.7	10.8	8.7	3.3	1.3	0.5	0.5
30 Mar.	12.3	12.0	4.1	0.9	3.9	1.1	1.1
14 Apr.	128.6	133.0	128.0	103	67.7	48.3	3.4
15 Aug.	5.5	5.31	5.6	2.7	0.3	0.1	0.1
16 Nov.	6.9	6.4	5.7	4.9	4.9	4.8	4.8
PAR ($\mu mol/s/m^2$)							
29 Feb.	1029	162	58	22	6.9	0.88	0.20
30 Mar.	106	24	8.7	3.8	1.6	0.51	0.05
14 Apr.	778	318	107	29	11	4.6	1.4
15 Aug.	1534	721	355	196	83	22	0.01
16 Nov.	82	16	4.2	1.2	0.40	0.14	0.05
Salinity (mg/l)							
30 Mar.	467	496	693	706	704	731	842
14 Apr.	652	659	658	759	661	669	779
15 Aug.	728	712	693	702	713	817	1240
16 Nov.	701	694	693	698	697	698	701
Conductivity ($\mu S/cm$)							
30 Mar.	883	927	1306	1323	1316	1361	1570
14 Apr.	1240	1236	1240	1245	1247	1258	1482
15 Aug.	1368	1299	1304	1309	1325	1523	2320
16 Nov.	1306	1300	1294	1310	1310	1310	1311

Secchi disk	
Date	D (m)
29 Feb.	2.47
30 Mar.	1.38
14 Apr.	1.74
16 Nov.	1.60

The temperature values increased with the depth in February, while in March these values decreased to the depth of 4 m and then progressively increased to the lake bottom. In November we observed an „autumn mixing" of the lake waters, when the temperature registered was close to 9ºC at all depths. This „autumn mixing" was also observed in the trend of the other physicochemical parameters measured.

The pH values varied between 6.6 and 8.25, the smallest values having been registered in the lowest horizons of the water. The acid character of this level was probably due to the presence of H_2S resulted from the decomposition of organic matter. The water sampled from this level had a pronounced odour of hydrogen sulphide. The pH values were smaller in summer than the ones measured in the other seasons. This decreasing may be due to a higher input of organic matter transported by the four lake tributaries.

The oxygen concentration (mg/l) decreased with the depth in all seasons, which was also the case of the photosynthetically available radiation (PAR). The salinity and conductivity values increased with depth in all seasons.

In the prospected pelagic station two species of Copepoda (*Cyclops vicinus* Uljanin, 1875 and *Mesocyclops* (*Thermocyclops*) *crassus* Rehberg, 1880) [1]; four species of Cladocera (*Daphnia galeata* Sars, 1864; *Bosmina longirostris* O.F.M., 1785; *Ceriodaphnia pulchella* Sars, 1862; *Chydorus sphaericus* O.F.M., 1785) [5] and the predatory larva of *Chaoborus flavicans* (Diptera) were identified. Their density was seasonally changing (Table 2).

Density of the Copepod species increased from winter to summer and then decreased in autumn. The maximum density of the Cladoceran species was registered in autumn (220,809 ind./m^3, November 16, 2000, 2 m depth). These facts can be correlated with the increasing insolation and water temperature, conditions of the phytoplankton development (primary production), the main source of nutrition for the most zooplanktonic organisms. The alga *Ceratium hirundinella* was very abundant during summer and autumn in all water levels.

Density of the larval stages of copepods (nauplia and copepodid) increased from winter to summer, then decreased in autumn.

It is known that cladocerans form an important part of the nurture of copepods [2]. The same aspect was observed in the Ştiucii Lake, where a great difference between the densities of copepods and cladocerans was registered. This difference can relate about the competition between the two groups.

The predatory larva of *Chaoborus flavicans* (Diptera) appeared at depths between 6 and 6.5 m. Its density seems to be insignificant, at least during daylight. At the same depth a relatively high density of the other zooplanktonic organisms was observed, in contrast with the overlying horizons, probably due to other causes, such as anoxic conditions or lack of nutrients, and not because of the presence of this predator.

Table 2

Density of zooplankton (individuals/m^3) in the Ştiucii Lake during 2000

Date	Depth (m)	Nauplia	Copepodid	*C. vicinus*	*M. (Th.) crassus*	*D. galeata*	*B. longirostris*	*C. pulchella*	*C. sphaericus*	*Ch. flavicans*
29 Feb.	0	633	100	200	–	–	100	–	–	–
	1	1867	133	267	–	–	533	–	–	–
	2	80813	1500	1633	–	–	1867	–	–	–
	3	109618	8300	8000	–	33	3600	–	–	–
	4	85614	24801	11467	–	–	5000	–	–	–
	5	19680	10000	7000	–	33	4600	–	–	–
	6	16547	9752	6432	–	–	3466	–	–	167
30 Mar.	0	24267	2700	1000	–	–	900	–	–	–
	1	27601	23601	10400	–	67	3200	–	33	–
	2	51208	29601	10720	–	100	6700	–	33	–
	3	59209	50408	20801	–	33	4100	–	33	–
	4	54409	40807	17334	–	33	2200	–	–	–
	5	30801	16801	13280	–	–	950	–	–	–
	6	34406	28004	5680	–	–	3200	–	-	67
14 Apr.	0	74133	7771	8178	–	233	6200	–	33	–
	1	89614	45202	29069	–	167	4500	–	–	–
	2	201608	46002	25067	–	100	8400	–	33	–
	3	91215	39470	21120	–	100	4400	–	–	–
	4	171227	58409	24267	–	33	1800	–	–	–
	5	86614	30801	26401	–	100	2000	–	–	–
	6	18134	19201	10934	–	–	267	–	–	–
15 Aug.	0	2250	6300	–	6150	100	550	1850	500	–
	1	19600	15600	–	16800	150	7467	20267	50	–
	2	8700	4200	–	8100	750	7800	12300	–	–
	3	73212	24601	–	34201	150	34801	73212	–	–
	4	63610	30000	–	19800	750	28801	68411	–	–
	5	127220	31201	–	28801	5850	28801	108017	–	–
	6	147623	38402	–	41402	4950	19201	88814	–	200
16 Nov.	0	7800	16501	1800	8100	1800	25501	300	–	–
	1	11100	36601	3300	18000	6600	187207	–	–	–
	2	3900	46202	6300	15600	9600	220809	600	–	–
	3	2400	19201	5400	16801	4800	106204	–	–	–
	4	2400	24000	18001	9300	6900	102004	–	–	–
	5	3000	25801	78903	0	9600	127205	–	–	–
	6	7800	56402	227409	0	15601	142806	–	–	–

In the seasonal succession of a zooplanktonic community the species are excluding one another, one or two species being dominant at a given period [3]. The same phenomenon was observed in the Ştiucii Lake where *Bosmina longirostris* had a higher density than *Daphnia galeata*. Specimens of *Ceriodaphnia pulchella* appeared in summer in a very high density, and disappeared in autumn. *Mesocyclops* (*Thermocyclops*) *crassus* replaced *Cyclops vicinus* in summer, but the two species appeared together in autumn. The reason why *Mesocyclops* (*Thermocyclops*) *crassus* appeared only in summer and autumn is the fact that it is a thermophilous species.

The absence of *Cyclops vicinus* in summer may be explained by the competition between the two species.

Population structure of the zooplanktonic organisms is presented in Tables 3 and 4.

Table 3

Fertility and fecundity of the Cladoceran species in the Ştiucii Lake during 2000

Date	Depth (m)	*Daphnia galeata*			***Bosmina longirostris***			***Chydorus sphaericus***		
		Ovigerous (%)	Non-ovigerous (%)	Eggs/ female	Ovigerous (%)	Non-ovigerous (%)	Eggs/ female	Ovigerous (%)	Non-ovigerous (%)	Eggs/ female
29 Feb.	0	0	100	0	0	100	0	0	0	0
	1	0	100	0	31	69	2	0	100	0
	2	0	100	0	20	80	2	0	100	0
	3	0	100	0	8	92	2	100	0	2
	4	0	0	0	12	88	2	0	0	0
	5	0	100	0	13	87	1	0	0	0
30 Mar.	0	0	0	0	22	78	2	0	100	0
	1	0	100	0	16	84	2	0	100	0
	2	0	100	0	52	48	2	0	100	0
	3	0	100	0	39	61	2	0	100	0
	4	100	0	0	68	32	2	0	0	0
	5	0	0	0	32	68	2	0	0	0
	6	0	0	0	34	66	2	0	0	0
14 Apr.	0	0	100	0	7	93	4	0	100	0
	1	0	100	0	56	44	4	0	100	0
	2	33	67	8	40	60	4	0	100	0
	3	0	100	0	62	38	3	0	100	0
	4	0	100	0	33	67	4	0	0	0
	5	33	67	6	65	35	5	0	0	0
	6	0	0	0	38	62	4	0	0	0
15 Aug.	0	100	0	2	0	100	0	0	100	0
	1	67	33	2	0	100	0	0	100	0
	2	40	60	2	0	100	0	0	100	0
	3	0	100	0	0	100	0	0	100	0
	4	20	80	2	0	100	0	0	100	0
	5	0	100	0	0	100	0	28	72	2
	6	36	64	2	0	100	0	27	73	2
16 Nov.	0	34	66	2	5	95	2	0	100	0
	1	0	100	0	13	87	2	0	0	0
	2	0	100	0	12	88	2	0	100	0
	3	13	87	2	16	84	2	0	0	0
	4	4	96	2	33	67	2	0	0	0
	5	31	69	2	48	52	2	0	0	0
	6	54	46	2	56	44	2	0	0	0

Table 4

Sex ratio of the Copepod species in the Ştiucii Lake

		Cyclops vicinus		***Mesocyclops* (*Th.*) *crassus***	
Date	Depth (m)	Males (%)	Females (%)	Males (%)	Females (%)
29 Feb.	0	50	50	–	–
	1	88	12	–	–
	2	67	33	–	–
	3	64	36	–	–
	4	63	37	–	–
	5	66	34	–	–
	6	–	–	–	–
30 Mar.	0	60	40	–	–
	1	70	30	–	–
	2	65	35	–	–
	3	53	47	–	–
	4	42	58	–	–
	5	39	61	–	–
	6	56	44	–	–
14 Apr.	0	16	84	–	–
	1	16	84	–	–
	2	31	69	–	–
	3	36	64	–	–
	4	40	60	–	–
	5	34	66	–	–
	6	41	59	–	–
15 Aug.	0	–	–	78	22
	1	–	–	67	33
	2	–	–	61	39
	3	–	–	56	44
	4	–	–	62	38
	5	–	–	54	46
	6	–	–	55	45
16 Nov.	0	50	50	4	96
	1	64	36	22	78
	2	71	29	27	63
	3	56	44	52	48
	4	42	58	35	65
	5	29	71	0	0
	6	25	75	0	0

The *Daphnia galeata* specimens reached the maximum fecundity and fertility in summer, when a large number of females were found carrying an average of four eggs per egg-carrying female. *Bosmina longirostris* reached this maximum in April, when the average number of eggs per egg-carrying female was four. In August, no *Bosmina longirostris* egg-carrying females were found, but they reappeared again in November.

In the case of *Mesocyclops* (*Thermocyclops*) *crassus*, a net dominance of the males at every level was observed during summer, while in autumn the ratio changed in the favour of females. The same aspect was observed for *Cyclops vicinus*. In this case, the males were more abundant than females over the whole year, with the exception of the April 14, 2000 sampling, when females numerically dominated the *Cyclops vicinus* males.

Conclusions. 1. Density of the copepod species increased from winter to summer, and decreased then in autumn. The maximum density of cladoceran species was observed in autumn, when an explosion of *Bosmina longirostris* specimens was registered. A great difference between the densities of copepods and cladocerans was registered.

2. In the seasonal succession of the zooplankton community, *Mesocyclops* (*Thermocyclops*) *crassus* replaced *Cyclops vicinus* in summer, but the two species appeared together in autumn. Summer was the single season when *Ceriodaphnia pulchella* appeared.

3. The fertility and fecundity maximum of the cladoceran species was registered in spring (*Bosmina longirostris*) and summer (*Daphnia galeata*), when the average number of eggs per egg-carrying female was four. The calculated sex ratio of the two copepod species showed a dominance of males for all seasons.

4. The predator *Chaoborus flavicans* appeared only in deep waters. The presence of this larva seems to have an insignificant influence on the zooplanktonic community.

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BIOTRANSFORMAREA DIGITOXINEI DE CĂTRE CELULELE DE *DIGITALIS LANATA* IMOBILIZATE ÎN ALGINAT DE CALCIU

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SUMMARY. - Biotransformation of Digitoxin by Cells of D i g i t a l i s l a n a t a Immobilised in Calcium Alginate. For biotransformation of digitoxin, two *Digitalis lanata* cell lines were used: cell line 15 (hormone-dependent) and cell line 78 (habituate, hormone-independent), the cells of which were immobilised in calcium alginate. The digitoxin was dissolved either in dimethylsulphoxide (DMSO) or in methanol and added to the culture media in different concentrations and at different growth stages of the immobilised cells. It was found that in comparison with the viability and growth of cell suspensions the viability of the immobilised cells was affected only to a little extent, but their growth was much slower. The factor that had a strongly negative influence on both viability and growth of the immobilised cells was one of the two solvents, namely the methanol. It was also found that in all experimental variants more than 90% of the digitoxin was converted into purpurea glycoside A (PgA). The rate of conversion was influenced mainly by the cell line. Thus, cell line 78 biotransformed digitoxin into PgA during the first 5 days, but cell line 15 did so only after 10 days. PgA was excreted in the culture medium in proportions ranging from 42 to 75%. It was pointed out as well that, in certain cases, the *D. lanata* cells are able to catalyse, through their enzyme systems, not only the glucosylation, but also the hydroxylation of digitoxin. Thus, the cell line 15 was able to hydroxylise digitoxin, converting it into gitoxin (in a proportion of about 3% relative to the amount of digitoxin), irrespective of the nature of the solvents used. Contrarily, cell line 78 biotransformed digitoxin into gitoxin (in a proportion of about 5%) only when methanol was the solvent.

Cormofitele constituie una dintre cele mai importante resurse naturale. Ele furnizează nu numai alimente, fibre şi lemn, ci şi numeroşi compuşi chimici, aşa cum sunt uleiurile, aromatizantele, pigmenţii şi principiile farmacologic active. Cu toate că plantele sunt resurse care se pot reînnoi, deja anumite specii sunt foarte greu de obţinut în cantităţi suficiente pentru a satisface cerinţele din ce în ce mai mari. Distrugerea habitatelor naturale, precum şi dificultăţile în cultivarea anumitor plante au cauzat reducerea acestor resurse. Mulţi dintre astfel de compuşi naturali pot fi

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sintetizaţi pe cale chimică din petrol sau din cărbune. Totuşi, limitarea economică, precum şi poluarea care însoţeşte acest tip de sinteză au condus inevitabil la amplificarea cercetărilor din domeniul culturilor celulare vegetale, în scopul producerii unor compuşi naturali valoroşi.

Culturile de ţesuturi şi celule vegetale oferă o promiţătoare alternativă, aşa cum specificam mai sus, pentru obţinerea unor cantităţi extrem de mari de metaboliţi secundari valoroşi din punct de vedere biologic.

Avantajul utilizării culturilor celulare vegetale în scopul obţinerii de principii biologic active este multiplu şi constă, printre altele, şi în faptul că astfel de compuşi sunt obţinuţi în condiţii controlate de mediu, independent de modificările climatice şi de condiţiile din sol. De asemenea, produşii izolaţi în astfel de condiţii sunt lipsiţi de contaminări cu microbi sau cu insecte [30].

Culturile celulare vegetale induse din cormofite sunt, în unele cazuri, capabile să sintetizeze metaboliţi secundari în cantităţi egale sau chiar mai mari decât plantele intacte. De asemenea, astfel de culturi posedă, prin setul de enzime pe care îl deţin, capacitatea de a cataliza biotransformarea anumitor substraturi naturale sau sintetice în compuşi biologic activi valoroşi.

Reacţiile de biotransformare sau de bioconversie, realizate prin intermediul culturilor de celule vegetale, sunt de cele mai multe ori stereospecifice şi implică, în majoritatea cazurilor, adiţia sau îndepărtarea anumitor grupări chimice. Dintre aceste reacţii, cele mai importante sunt glucozilarea şi hidroxilarea. Au fost astfel utilizaţi ca substraturi pentru bioconversie o serie de compuşi cum sunt: terpenoizii, steroizii, fenolii, alcaloizii etc. [3, 28, 29, 34, 36].

Digitoxina a fost prima glicozidă cardiotonică, de natură steroidică, folosită în experimentele de biotransformare cu suspensii celulare induse din cormofite [33], fiind în continuare investigată intens de către mai mulţi cercetători, care au utilizat culturi de ţesuturi şi celule fie de *Digitalis purpurea* [16, 31], fie de *D. lanata* [2, 3, 10, 11, 15, 20 - 22, 25, 29], în scopul obţinerii de digoxină. Totuşi, cele mai mari producţii au fost obţinute în urma biotransformării metildigitoxinei în metildigoxină [1]. Astfel, utilizând culturi celulare de *D. lanata*, A l f e r m a n n şi colab. [5] au reuşit să obţină 500 g de β-metildigoxină pe o perioadă de producţie de cca 3 luni, într-o staţie pilot de 200 litri.

Imobilizarea celulelor vegetale, respectiv a celulelor cormofitelor cultivate *in vitro*, este considerată importantă pentru cercetare şi producţie datorită mai multor avantaje, faţă de sistemul celulelor aflate sub formă de suspensie. Astfel, includerea (încapsularea) celulelor poate conduce la crearea unui microclimat (micromediu) asemănător cu cel existent în ţesuturile plantelor intacte, sau la condiţii care provoacă încetinirea creşterii celulelor. În plus, contactul fizic dintre celule poate stabili comunicarea intercelulară. Imobilizarea permite prelungirea utilizării biomasei la o densitate celulară mare, garantează separarea biomasei celulare de mediul de cultură,

iar în final celulele imobilizate pot fi utilizate numai ca şi biocatalizatori. De asemenea, se poate produce (sau induce) eliminarea compuşilor din celule în mediu, ceea ce simplifică izolarea lor şi face posibilă rentabilizarea biocatalizatorilor [28]. Metodele generale pentru imobilizarea celulelor vegetale prevăd includerea (înglobarea) lor în matrici polimerice, precum şi imobilizarea pe suporturi insolubile aşa cum sunt: plasele de nylon, spuma de poliuretan, fibre goale şi particule preactivate cu glutaraldehidă [26]. Dintre metodele de imobilizare citate mai sus, includerea celulelor vegetale în gel de alginat de calciu este metoda cel mai des utilizată, deoarece este simplă şi reproductibilă şi poate conduce la mărirea capacităţii de biotransformare. De exemplu, celulele de *Digitalis lanata* imobilizate în alginat de calciu au hidroxilat glicozida cardiotonică β-metildigitoxina în β-metildigoxină, la o rată de 9 mg/l/zi [4], iar celulele de *Mucuna pruriens,* imobilizate în acelaşi tip de gel, au orto-hidroxilat L-tirozina în L-DOPA, medicament antiparkinsonian, la o rată de 5 mg/l/zi [35].

În contextul celor relatate mai sus, în această lucrare s-au luat în studiu mai multe aspecte care privesc modul şi capacitatea de biotransformare a digitoxinei de către două linii celulare de *D. lanata* imobilizate în gel de alginat de calciu, precum şi creşterea şi viabilitatea acestor celule.

Material şi metode. *Suspensiile celulare*. Pentru efectuarea experimentelor au fost utilizate două linii celulare de *Digitalis lanata*. Linia 15, este o linie normală (hormon-dependentă) care a fost cultivată în mediul bazal M u r a s h i g e-S k o o g (MS) [27]. La acest mediu s-au adăugat: zaharoză (3%), cazeină hidrolizată (500 mg/l), acidul indolilacetic - AIA (1 mg/l) şi chinetină – K (2 mg/l). pH-ul mediului s-a stabilit înainte de autoclavare la valoarea de 5,7. Linia 78, o linie habituată (hormon-independentă), a fost cultivată în acelaşi mediu bazal MS la care s-a adăugat doar glucoză (3%). pH-ul mediului a fost stabilit înainte de autoclavare la valoarea de 5,5. Modul de selecţie şi de izolare a acestor linii au fost descrise într-o lucrare anterioară [9]. Transferul culturilor celulare s-a efectuat la un interval de 14 zile. Raportul dintre volumul inoculului şi cel al mediului proaspăt a fost de 1:6. Suspensiile celulare au fost crescute în vase conice de 300 ml (cu 40 ml mediu lichid/vas); vasele au fost agitate pe un agitator rotativ orizontal (100 rpm) şi menţinute la întuneric, la o temperatură de $25 \pm 1{,}5^{o}C$.

Procedeul de imobilizare a celulelor şi mediile utilizate pentru biotransformare. Celulele celor două linii de *D. lanata* au fost imobilizate în gel de alginat de calciu după metodele descrise de A l f e r m a n n şi colab. [4] şi de M o r r i s şi colab. [26]. În acest context, suspensiile celulare de *D. lanata*, aflate în ziua a 9-a de cultură, au fost filtrate printr-o sită metalică cu ochiuri de 0,8 mm în diametru şi apoi recoltate pe o sită de nylon cu ochiuri de 100 µm în diametru. În continuare, o cantitate de 60 g biomasă celulară obţinută prin acest procedeu, din fiecare linie celulară, a fost amestecată, separat, cu 40 ml din mediul în care au fost cultivate

respectivele linii. Cele două tipuri de mixturi au fost apoi amestecate, separat, cu 100 ml de alginat de sodiu (4%) (lichefiat şi autoclavat în prealabil la 121°C, timp de 20 min.). Cele două linii celulare amestecate cu alginatul de sodiu au fost adăugate, separat, picătură cu picătură, cu ajutorul unor pipete fine, într-o soluție de $CaCl_2$ (100 mM). La contactul cu soluția de $CaCl_2$, picăturile de alginat de sodiu s-au gelificat şi s-au transformat în mici sfere de alginat de calciu în interiorul cărora erau înglobate celulele celor două linii. Pentru întărire, sferele de alginat au fost menținute încă două ore în soluția de $CaCl_2$, apoi au fost spălate de trei ori cu un mediu corespunzător celor două linii. În urma aplicării acestui procedeu au rezultat 200 ml de sfere (per linie celulară) cu un diametru de 3-4 mm, sfere care au conținut cca 0,2-0,3 g de biomasă celulară proaspătă.

În continuare, celulele imobilizate în gel de alginat (350 de sfere per vas) au fost transferate în vase conice de 300 ml, vase care conțineau 50 ml mediu de cultură. Compoziția mediilor nutritive utilizate pentru biotransformare este redată în Tabelul 1. Vasele utilizate pentru biotransformare au fost menținute pe un agitator orizontal în condițiile descrise mai sus. În toate cazurile, celulele imobilizate au fost menținute timp de 4 zile în mediul respectiv, mediu la care, după acest răstimp, s-a adăugat digitoxina. Pe grafice, momentul de introducere a digitoxinei în mediul de cultură al suspenilor celulare este notat ca fiind ziua 0 de cultură.

Adăugarea digitoxinei şi analiza cardenolidelor. Digitoxina a fost dizolvată fie în dimetilsulfoxid (DMSO), fie în metanol şi apoi a fost introdusă în mediul de cultură al celulelor imobilizate, prin filtrare sterilă. În cazul dizolvării ei în DMSO, digitoxina a fost adăugată în două variante: 1) într-o singură doză, de la început – ziua 0 (respectiv ziua a 4-a de cultură), într-o concentrație finală de 250 mg/l per vas de cultură – respectiv 1 ml DMSO/vas de cultură, astfel că în vasul de cultură, DMSO a avut o concentrație finală de 2%; 2) în 5 doze a câte 50 mg/l (concentrație finală per vas) fiecare, succesiv, din 3 în 3 zile, începând cu ziua 0 de cultură, dizolvată în 0,2 ml DMSO, astfel că la sfârşitul celor 15 zile cantitatea totală de digitoxină adăugată a fost de 250 mg/l, iar concentrația de DMSO de 2%.

În cazul metanolului, digitoxina a fost adăugată într-o singură variantă şi anume dizolvată în 0,5 ml metanol (concentrația finală în vas a fost de 50 mg/l) şi apoi suplimentată succesiv, din 3 în 3 zile, de 5 ori, astfel că la sfârşitul celor 15 zile de cultură concentrația totală de digitoxină adăugată a fost de 250 mg/l, iar aceea a metanolului de 2,5%.

Recoltările probelor (biomasă celulară şi mediu) s-au efectuat fie din 5 în 5 zile, fie numai la sfârşitul perioadei de cultură, respectiv la 15 zile. Pentru o mai bună înțelegere, toate variantele experimentale utilizate în biotransformarea digitoxinei sunt redate în Tabelul 1. Cardenolidele au fost extrase atât din substanța uscată cât şi din mediu, cu 20 ml metanol (70%), prin refluxare la 70°C timp de 20 min. şi dozate după metoda dată de J o n e s şi V e l i k y [18]. Identificarea glicozidelor cardiotonice

Tabel 1

Variantele experimentale utilizate în biotransformarea digitoxinei de către celulele de D i g i t a l i s l a n a t a imobilizate în gel de alginat

Varianta	Linia celulară imobilizată	Compoziția mediului utilizat pentru biotransformare	Adăugarea digitoxinei în mediul de cultură al celulelor imobilizate
M1 = martor pentru V1, V 5 şi V 6	15	MS cu 5% glucoză + AIA[a] (1 mg/l) + K[b] (2 mg/l)	-
M2 = martor pentru V2 şi V 7	15	MS cu 5% glucoză, lipsit de fitohormoni	-
M3 = martor pentru V3 şi V8	78	MS cu 7% glucoză, lipsit de fitohormoni	-
M4 = martor pentru V4 şi V9	78	Apă de robinet cu 7% glucoză, fără fitohormoni	-
V1	15	Similară cu aceea de la M1	Dgtx[c] (250 mg/l), dizolvată în 1 ml DMSO[d], a fost adăugată, în ziua a 4-a de cultură. Recoltarea probelor s-a efectuat la 5, 10 şi 15 zile după adăugarea Dgtx.
V2	15	Similară cu aceea de la M2	Adăugarea Dgtx şi recoltarea probelor s-au efectuat ca şi la V1.
V3	78	Similară cu aceea de la M3	Adăugarea Dgtx şi recoltarea probelor s-au efectuat ca şi la V1.
V4	78	Similară cu aceea de la M4	Adăugarea Dgtx şi recoltarea probelor s-au efectuat ca şi la V1.
V5	15	Similară cu aceea de la M1	Dgtx (50 mg/l), dizolvată în 0,2 ml DMSO, a fost adăugată, succesiv, din 3 în 3 zile, începând din ziua a 4-a de cultură, de 5 ori. Recoltarea probelor s-a efectuat după 15 zile de la introducerea Dgtx.
V 6	15	Similară cu aceea de la M1	Dgtx (50 mg/l), dizolvată în 0,5 ml metanol, a fost adăugată, succesiv, din 3 în 3 zile, începând din ziua a 4-a de cultură, de 5 ori. Recoltarea probelor s-a efectuat după 15 zile de la introducerea Dgtx.
V7	15	Similară cu aceea de la M2	Adăugarea Dgtx şi recoltarea probelor s-au efectuat ca şi la V5.
V8	78	Similară cu aceea de la M3	Adăugarea Dgtx şi recoltarea probelor s-au efectuat ca şi la V6.
V9	78	Similară cu aceea de la M4	Adăugarea Dgtx şi recoltarea probelor s-au efectuat ca şi la V5.

[a] AIA - Acid indolilacetic.
[b] K - Chinetină.
[c] Dgtx - Digitoxină.
[d] DMSO - Dimetilsulfoxid.

s-a realizat prin cromatografie în strat subțire, în urma comparării cromatografice a extractelor cu probele etalon. Pentru determinarea cantitativă, plăcile cromatografice au fost citite la 380 nm cu un densitometru CD 200. Curba etalon a fost realizată pentru concentrații de 0,2–0,6 µg digitoxină, digoxină, gitoxină, glicozidă purpurea A /spot.

Creşterea celulară. Pentru determinarea creşterii celulare, în cazul suspensilor celulare, biomasa celulară a fost recoltată prin filtrare la anumite intervale de timp (din 5 în 5 zile). Biomasa celulară obținută a fost uscată (la 60^{o}C timp de 24 de ore) şi cântărită. În cazul variantelor imobilizate, după decantarea şi filtrarea mediului, pentru obținerea biomasei celulare, sferele din alginat de calciu au fost dizolvate într-o soluție de citrat de sodiu (200 mM). Celulele eliberate au fost recoltate prin filtrare, uscate în aceleaşi condiții, şi cântărite. Substanța uscată şi mediul rezultat în urma filtrării au fost utilizate pentru determinarea calitativă şi cantitativă a glicozidelor rezultate în urma biotransformării digitoxinei. Rata creşterii celulare s-a exprimat în mg de substanță uscată/l/zi şi a fost calculată după formula: $S_1 – S_o/T_1 – T_o$, unde S_1 = substanța uscată recoltată la timpul 1 (5, 10 şi 15 zile - T_1); S_o = substanța uscată recoltată la timpul 0 (T_o – fiind fie momentul inoculării, fie momentul introducerii digitoxinei în mediu). Această determinare a fost efectuată după metoda lui S i n g e r [32].

Viabiliatea celulară. Viabilitatea celulelor celor două linii a fost determinată după metoda lui D o d d s şi R o b e r t s [12], modificată. Metoda se bazează pe procesul de respirație a celulelor, care, la nivel mitocondrial, reduc compusul incolor, clorura de 2,3,5-trifeniltetrazoliu (TTC), la formazan, produs de culoare roşie. Prezența coloraţiei roşii în celule dovedeşte viabilitatea lor. În cazul nostru, celulele recoltate din suspensii, precum şi cele imobilizate (secțiuni foarte fine din sferele de alginat) au fost spălate de 2-3 ori cu apă distilată şi apoi imersate într-o soluție tampon fosfat (pH 7,5) în care s-a dizolvat 0,8% TTC. În această soluție, suspensiile celulare au fost menținute timp de 2-3 ore, iar celulele imobilizate timp de 10 ore, după care au fost spălate şi numărate (300 de celule per probă). Observațiile au fost efectuate la microscopul optic. Viabilitatea s-a calculat după formula: nr. de celule colorate în roşu/nr. total de celule numărate x 100, şi s-a exprimat în procente.

Randamentul de biotransformare. Capacitatea celor două linii celulare de biotransformare a digitoxinei în alte glicozide steroidice a fost exprimată şi prin randamentul lor de biotransformare, calculat (în procente) după cantitatea totală de glicozide rezultată în urma biotransformării, față de cantitatea totală de digitoxină introdusă în mediu.

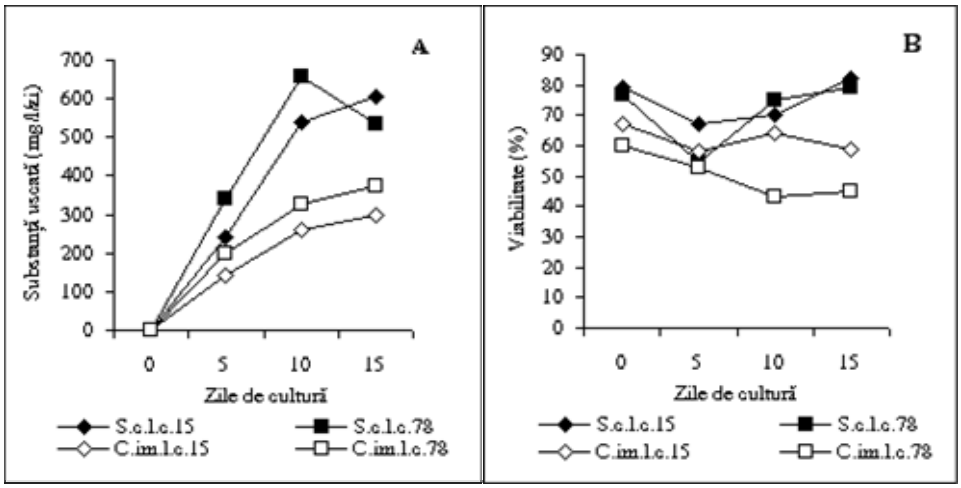
Rezultate şi discuții. *Creşterea şi viabilitatea celulară*. Liniile celulare 15 şi 78 de *Digitalis lanata*, selecționate de noi, şi-au păstrat aproape neschimbate, pe durata a doi ani de subcultivări succesive în acelaşi tip de mediu - MS, o serie de caracteristici cum sunt: creşterea celulară, modificarea pH-ului mediului de cultură,

raportul dintre substanța proaspătă şi cea uscată, precum şi intensitatea absorbției zaharozei din mediu. Dacă linia 15 este o linie celulară normală, care creşte numai într-un mediu în care sunt prezente auxina şi citochinina, linia 78 este o linie hormon-independentă, o linie care creşte foarte bine fără nici un adaos de hormoni vegetali. O astfel de linie poate fi încadrată în categoria culturilor celulare vegetale habituate. Se ştie că habituarea (sau habituația) este un proces progresiv de schimbare a metabolismului care apare la țesuturile sau celulele vegetale cultivate *in vitro*, considerându-se că acest proces are la bază mai degrabă modificări epigenetice decât genetice. Habituarea poate apare spontan, dar poate fi şi indusă prin manipularea factorilor de mediu, aşa cum este cazul variațiilor de temperatură (şoc termic). Ea se caracterizează prin pierderea dependenței celulelor vegetale cultivate *in vitro* față de: auxine (habituare auxinică); de citochinine (habituare citochininică); de orice tip de hormon vegetal (habituare hormonală), sau față de vitamine [24]. Deci, în cazul nostru, linia 78 poate fi considerată o linie celulară hormon-habituată.

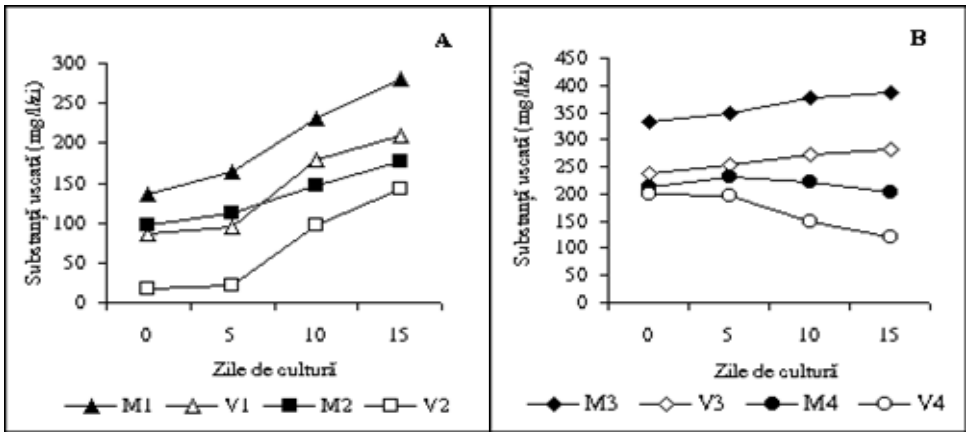
Imobilizarea celulelor celor două linii în alginat de calciu a influențat anumite procese fiziologice ale celulelor, aşa cum sunt creşterea şi viabilitatea. În primul caz, aceste modificări se reflectă, la ambele linii celulare, în special asupra ritmului de creştere al celulelor imobilizate, care este mult mai lent şi mai scăzut decât al celor aflate sub formă de suspensii. Astfel, dacă la suspensiile celulare creşterea atinge în ziua a 15-a de cultură o rată cuprinsă între 500 şi 600 mg substanță uscată/l/zi, la celulele imobilizate aceasta ajunge doar la valori cuprinse între 300 şi 400 mg substanță uscată/l/zi (Fig. 1A). Astfel de scăderi ale ratei de creştere, ca urmare a imobilizării celulelor, sunt curente şi diferă ca intensitate în funcție de strategiile utilizate pentru imobilizare [14]. În ceea ce priveşte viabilitatea celulară, aceasta este mai puțin afectată, ea oscilând între 60 şi 85% la suspensiile celulare, şi între 50 şi 78% la celule imobilizate (Fig. 1B).

Cultivarea celulelor imobilizate în medii cu o compoziție diferită de aceea a martorului a avut ca efect, la ambele linii celulare, inhibiția, mai mult sau mai puțin accentuată, atât a creşterii (Fig. 2A şi 2B) cât şi a viabilității celulelor (Fig. 3A şi 3B), aşa cum a fost cazul cultivării celulelor liniei 15 (M1) într-un mediu lipsit de hormoni (M2) şi a liniei 78 (M3) în apă de robinet (M4). Fenomenul este normal având în vedere că linia 15 a fost transferată într-un mediu lipsit de una din sursele esențiale necesare diviziunii şi creşterii celulelor – fitohormonii, iar linia 78 a fost cultivată într-un mediu cu o compoziție total diferită de mediul MS, şi anume apa de robinet.

Utilizarea celor doi solvenți, DMSO şi metanolul, a avut ca scop nu numai dizolvarea digitoxinei pentru a putea fi introdusă în mediul de cultură, ci şi acela de a urmări dacă aceste substanțe au anumite efecte asupra procesului de biotransformare. Într-un prim experiment, DMSO (în care a fost dizolvată digitoxina) a fost adăugat într-o concentrație de 2%, începând cu ziua 0 (respectiv ziua a 4-a) de cultură, în mediul celor două linii celulare (variantele V1 şi V2 - linia 15, şi variantele V3 şi



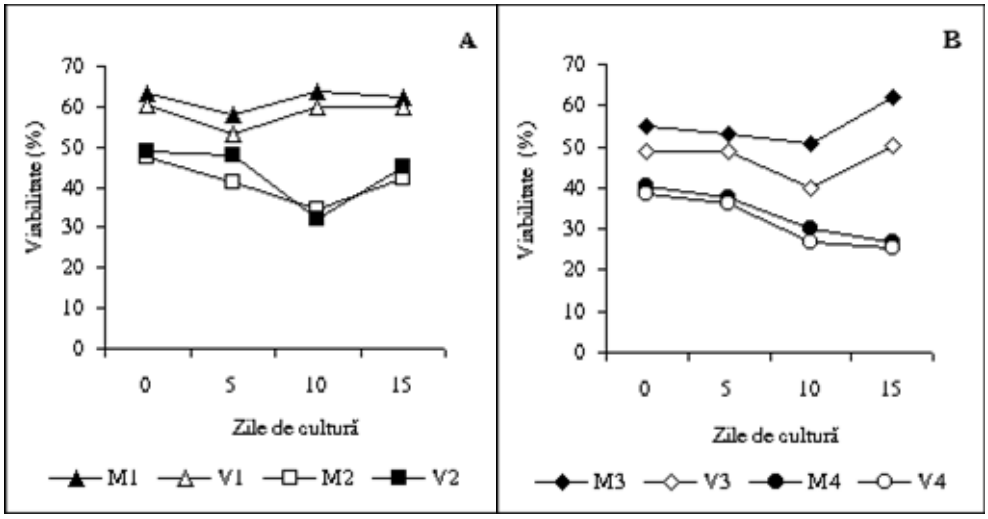
F i g. 1. *Creşterea (A) şi viabilitatea (B) celulelor de* Digitalis lanata *cultivate* in vitro *sub formă de suspensii şi imobilizate în alginat de calciu.* S.c.l.c.15 - Suspensie celulară, linia celulară 15. C.im.l.c.15 - Celule imobilizate, linia celulară 15. S.c.l.c.78 - Suspensie celulară, linia celulară 78. C.im.l.c.78 - Celule imobilizate, linia celulară 78.



F i g. 2. *Efectul compoziţiei mediului de cultură asupra creşterii liniei celulare 15 (A) şi a liniei celulare 78 (B) de* Digitalis lanata *imobilizate în alginat de calciu.* M1, M2, M3, M4 – Martori. V1, V2, V3, V4 - Variante (explicaţiile se găsesc în Tabelul 1).

V4 - linia 78). Acest adaos a avut ca efect, mai ales spre finalul perioadei de cultură, o inhibiţie mai mult sau mai puţin accentuată a ratei de creştere, în funcţie de linia celulară şi de mediul utilizat pentru biotransformare. Astfel, în cazul liniei 15, scăderea ratei creşterii celulare la V1, faţă de M1, a fost de cca 30% şi numai de 18% la V2, faţă de M2. La linia 78, efectele au fost similare, creşterea celulelor din varianta V3 fiind mai scăzută (cu 40%) decât aceea a celulelor din varianta V4

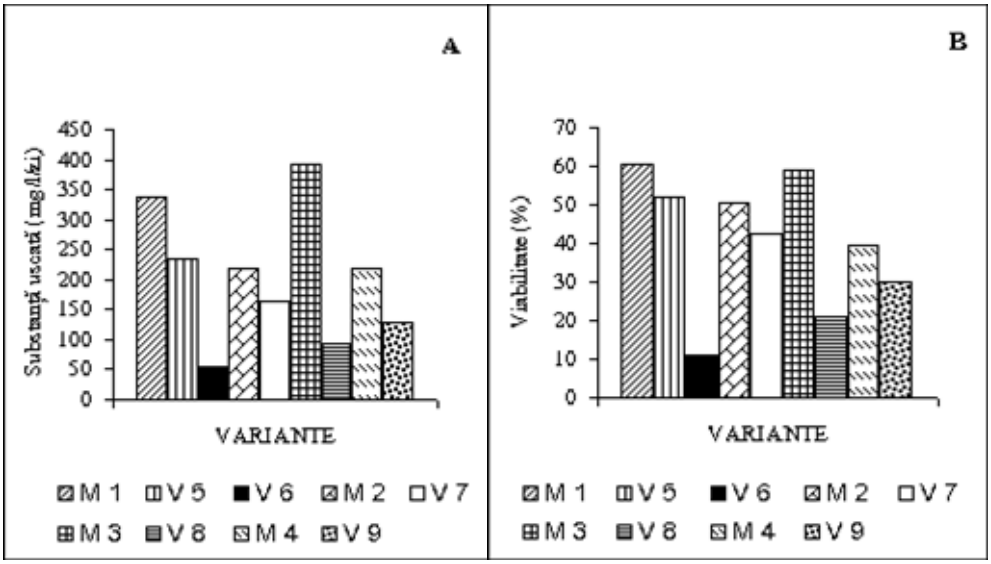
(cu 30%) (Fig. 2A şi 2B). Totuşi, viabilitatea celulelor a fost mult mai puţin afectată de adaosul de DMSO, aceasta fiind însă inhibată puternic de compoziţia mediilor în care au fost crescute cele două linii celulare (Fig. 3A şi 3B).



F i g. 3. *Efectul compoziţiei mediului de cultură asupra viabilităţii celulelor liniei 15 (A) şi a celulelor liniei 78 (B) de* Digitalis lanata *imobilizate alginat de calciu*. M1, M2, M3, M4 – Martori. V1, V2, V3, V4 -Variante (explicaţiile se găsesc în Tabelul 1).

În majoritatea cazurilor, adăugarea unor solvenţi organici sau detergenţi în mediul de cultură al celulelor vegetale are ca scop permeabilizarea plasmalemei şi tonoplastului. De cele mai multe ori se observă că în urma unui astfel de tratament, are loc o scădere drastică a viabilităţii şi creşterii celulelor vegetale cultivate *in vitro*. După B r o d e l i u s [7], cel mai probabil este că pierderea viabilităţii şi inhibiţia creşterii celulare nu se datorează toxicităţii agentului permeabilizator, ci distrugerii celor două membrane plasmatice şi, implicit, a compartimentelor celulare, proces care conduce la eliminarea intracelulară a unor substanţe toxice, aşa cum sunt proteazele şi fenolii. În acest context, D ö r n e n b u r g şi K n o r r [13] au arătat că în cazul unor celule vegetale tratate cu diferiţi detergenţi, cele mai afectate sunt fosfolipidele din membrane, respectiv fosfatidilcolina şi fosfatidiletanolamina, care au fost convertite la acidul fosfatidic, probabil prin acţiunea fosfolipazei D. Totuşi, în cazul liniilor celulare de *Digitalis lanata*, 15 şi 78, izolate de noi şi imobilizate în alginat de calciu, adăugarea solventului DMSO nu a avut efecte drastice asupra viabilităţii şi creşterii celulare. Un fenomen similar a fost constatat şi de B r o d e l i u s şi N i l s s o n [8] la culturile celulare de *Catharanthus roseus* imobilizate în alginat de calciu.

Introducerea de DMSO (în care era dizolvată digitoxina) în mediul de cultură al celor două linii celulare (V5, V7 şi V9) în doze mai mici (0,4%) şi succesive, de 5 ori, din 3 în 3 zile, a avut, după 15 zile de cultură, efecte similare cu cele constatate în primul experiment (Fig. 4A şi 4B). În schimb, utilizarea metanolului ca solvent al digitoxinei (V6 şi V8), în aceleaşi condiţii descrise mai sus, dar la o concentraţie a dozelor de 0,5%, a condus la inhibarea drastică (cu peste 60%) a ambilor parametri fiziologici luaţi în studiu, viabilitate şi creştere celulară. Totuşi, linia habituată 78 s-a dovedit a fi mult mai rezistentă la acţiunea acestui compus chimic, deoarece proporţia în care a scăzut atât viabilitatea cât şi creşterea celulelor a fost de cca 30% faţă de martor. Aspecte asemănătoare au fost remarcate şi în cazul experimentelor de biotransformare a digitoxigeninei efectuate cu celule de *Daucus carota* imobilizate în alginat de calciu. Astfel, J o n e s şi V e l i k y [17] au observat că la concentraţia de 2%, metanolul a avut ca efect inhibarea ratei respiraţiei celulare cu peste 50%.



F i g. 4. *Influenţa solventului digitoxinei asupra creşterii (A) şi viabilităţii (B) celulelor de* Digitalis lanata *imobilizate în alginat de calciu*. M1, M2, M3, M4- Martori. V5, V6, V7, V8, V9 - Variante (explicaţiile se găsesc în Tabelul 1).

Biotransformarea digitoxinei. În urma consultării datelor din literatura de specialitate aflată la dispoziţie, am remarcat că toate experimentele de biotransformare, realizate cu celule de *Digitalis lanata* aflate sub formă de suspensii sau imobilizate în gel de alginat, au vizat de la început aspectul practic, şi anume acela de a obţine cantităţi cât mai mari de digoxină sau metildigoxină, principalii compuşi activi care

intră în compoziţia medicamentelor utilizate în tratamentul unor boli cardio-vasculare [1 - 5, 20, 22]. Totuşi, deşi rezultatele obţinute încă din anii ’80 [4] cu celule de *D. lanata* imobilizate în alginat de calciu, celule utilizate în reacţiile de biotransformare, în special a metildigitoxinei în metildigoxină, au fost deosebit de încurajatoare, după această dată nu am mai întâlnit astfel de abordări. De asemenea, între anii 1986 – 1992, echipa condusă de K r e i s [15, 20, 21] anunţa izolarea unei linii celulare de *D. lanata* capabilă de a supravieţui într-un mediu MS lipsit de fitohormoni şi de a biotransforma digitoxina în digoxină şi, în special, în deacetillanatozida C, în cantităţi deosebit de mari. Ca şi în primul caz, după anul 1992, în literatura de specialitate nu a mai apărut nici o altă dată care să ateste şi să demonstreze eficacitatea utilizării acestui tip de experimente.

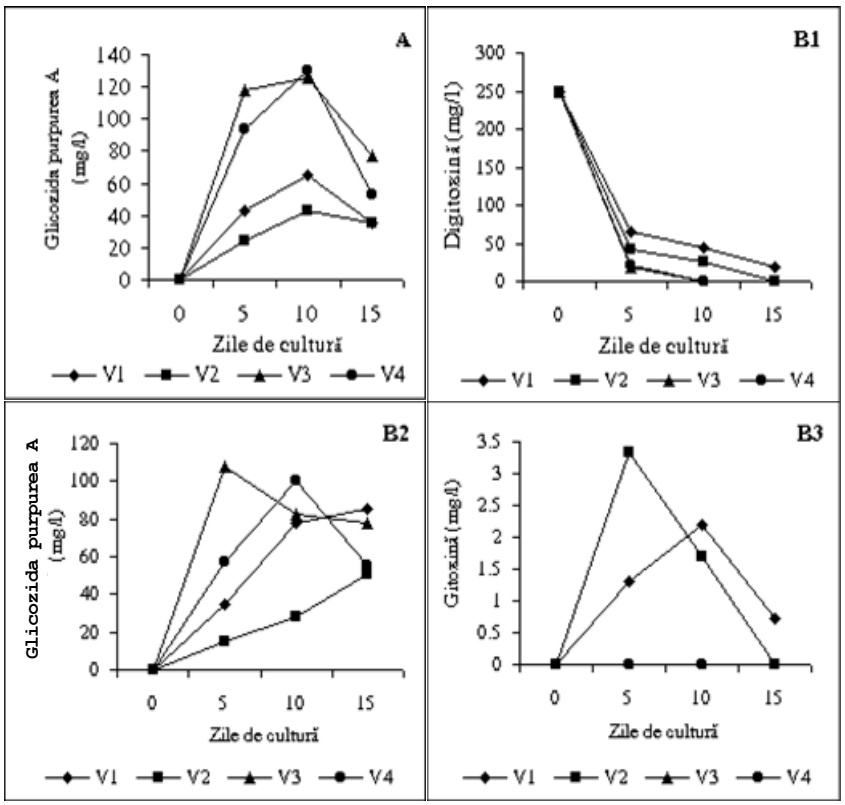
În contextul celor relatate mai sus, în cadrul experimentelor efectuate de noi, am abordat mai multe aspecte. În primul rând am încercat să selecţionăm linii celulare de *D. lanata* cu însuşiri metabolice deosebite, mai ales în ceea ce priveşte capacitatea de biotransformare a digitoxinei. Astfel, am reuşit să izolăm două linii celulare: linia 15 – o linie normală, hormon-dependentă şi o linie hormon-independentă (hormon-habituată) – linia 78. Ambele linii se caracterizează prin trăsături specifice distincte, atât în ceea ce priveşte metabolismul primar, cât şi cel secundar. În continuare, aceste linii celulare au fost imobilizate în alginat de calciu şi utilizate în toate experimentele de biotransformare a digitoxinei.

În scopul determinării capacităţii de biotransformare a digitoxinei de către cele două linii celulare luate în studiu a fost testată influenţa mai multor factori, şi anume: compoziţia mediului de cultură utilizat pentru biotransformare; natura solvenţilor în care a fost dizolvată digitoxina - DMSO şi metanol; concentraţia digitoxinei şi etapele în care aceasta a fost adăugată la mediul de cultură al celor două linii celulare.

Rezultatele obţinute confirmă că digitoxina dizolvată în DMSO şi introdusă de la început (în ziua 0, respectiv în ziua a 4-a de cultură) în mediul de cultură al celor două linii celulare, şi în cantitatea maximă utilizată de noi (250 mg/l), a fost biotransformată, aproape în totalitate, în glicozida purpurea A (GpA), indiferent de linia celulară utilizată şi de compoziţia mediului de cultură, încă din ziua a 10-a de cultură (Fig. 5 A). Transformarea digitoxinei în GpA este de fapt un proces de glucozilare, proces care constă în adiţia unei molecule de glucoză la lanţul glucidic (D-D-D-; D = β-D-digitoxoză) al agliconului digitoxinei şi este catalizat de o glucoziltransferază [11, 19]. În acest sens, linia celulară care a prezentat capacitatea maximă de bioconversie s-a dovedit a fi linia celulară 78, linie care, indiferent compoziţia mediului de cultură, încă din ziua 5-a a biotransformat digitoxina în GpA, într-o proporţie de peste 85% (V3 şi V4) (Fig. 5A). Ambele linii celulare au stocat intracelular o cantitate relativ mare de GpA din care o parte (peste 45%) a fost excretată în mediul de cultură. Acest tip de reacţie de biotransformare a digitoxinei

în GpA a mai fost semnalată şi în alte cazuri unde au fost utilizate culturi celulare de *D. lanata* aflate sub formă de suspensii sau imobilizate în alginat de calciu [2, 4, 10, 11, 16, 20, 21]. Astfel, A l f e r m a n n şi colab. [2] au arătat, încă din anul 1977, că tiparul biotransformării digitoxinei catalizate de către culturile celulare de *D. lanata* depinde de suşa sau linia celulară utilizată în experimente. În acest sens, una dintre suşele selectate de autori (72L) a glucozilat digitoxina la glicozida purpurea A. În continuare, autorii au constatat că GpA a fost acetilată, de către aceeaşi linie, la lanatozida A, compus care a fost apoi hidroxilat la lanatozida C. Totuşi, principala reacţie a fost hidroxilarea în poziţia C-12 a GpA, reacţie care a condus la formarea deacetillanatozidei C. În experimentele anterioare efectuate de noi cu culturi celulare de *D. lanata,* principalul compus rezultat în urma biotransformării digitoxinei a fost tot GpA. Alături de aceasta au mai fost însă detectate şi alte glicozide care au rămas neidentificate din cauza lipsei substanţelor etalon [10].

În cadrul prezentului studiu, am constatat că după introducerea digitoxinei în condiţiile descrise mai sus, aceasta a fost biotransformată într-un ritm destul de intens. Astfel că după 5 zile, în mediul de cultură al celor două linii celulare imobilizate, din totalul de 250 mg/l digitoxină au mai rămas aproximativ 75 mg/l, cantitate care a fost apoi bioconvertită treptat şi total în GpA până în ziua a 15-a de cultură (Fig. 5B1). Aşa cum arătam mai sus, o mare parte din acest compus rezultat în urma biotransformării digitoxinei a fost stocată în celule. Totuşi, este de subliniat că o cantitate destul de mare din totalul de GpA a fost excretată în mediu (Fig. 5 B2). Eliminarea a fost rapidă, linia celulară 78 dovedindu-se a fi cea mai prolifică şi în acest sens (V3 şi V4). Deşi nu a fost urmărit în mod expres, credem că fenomenul de excreţie constatat la celulele celor două linii, 15 şi 78, se datorează în principal permeabilizării membranelor plasmatice de către DMSO, solvent care a fost introdus în mediu împreună cu digitoxina, într-o concentraţie de 2%. Această afirmaţie se bazează în primul rând pe experimentele anterioare efectuate de noi cu suspensii celulare de *D. lanata,* în care, pentru introducerea digitoxinei în mediul de cultură am folosit DMSO într-o concentraţie de numai 0,75%. Cantitatea de GpA (rezultată în urma biotransformării digitoxinei) eliminată în mediu a fost mult mai mică decât aceea din prezenta lucrare, ea fiind cuprinsă între 7 şi 10% din total [10]. De asemenea, în urma experimentelor efectuate cu suspensii celulare induse din trei specii, *Catharanthus roseus*, *Chenopodium rubrum* şi *Thalictrum rugosum*, B r o d e l i u s [7] a observat că pentru eliminarea unei cantităţi de 50% din metaboliţii secundari stocaţi în celulele speciilor respective, au fost necesare următoarele concentraţii de DMSO: 3% pentru *C. roseus*, 10% pentru *C. rubrum* şi 13% pentru *T. rugosum*. Deci, este clar că permeabilizarea membranelor celulare depinde în cea mai mare măsură de trei factori, şi anume: de specie, de natura şi de concentraţia solventului. Astfel, este posibil ca suspensiile celulare de *D. lanata*, utilizate de noi, să fie mult mai sensibile la acţiunea DMSO.



F i g. 5. *Influența compoziției mediului de cultură asupra capacității de biotransformare a digitoxinei de către celulele liniilor 15 şi 78 de* Digitalis lanata, *imobilizate în alginat de calciu.* A – Glicozida purpurea A rezultată în urma biotransformării digitoxinei şi acumulată în celule. B1 – Cantitatea de digitoxină rămasă în mediu. B2 şi B3 – Cardenolidele rezultate în urma biotransformării digitoxinei şi excretate în mediul de cultură. V1, V2, V3, V4 - Variante (explicațiile se găsesc în Tabelul 1).

Majoritatea produşilor secundari sintetizați de către suspensiile celulare vegetale se acumulează intracelular, fiind stocați în diferite compartimente (în principal în vacuolă). Totuşi, este posibil ca să se obțină o cantitate mult mai mare de astfel de compuşi, dacă aceştia sunt secretați în mediul de cultură al celulelor, deoarece, în majoritatea cazurilor, metaboliții secundari acumulați intracelular inhibă însăşi sinteza lor printr-un mecanism de reglare de tip "feed-back".

Pentru majoritatea sistemelor de celule vegetale imobilizate este esențial ca o cantitate semnificativă din compuşii acumulați în celule să fie eliminată în mediu. Când produsul nu este eliminat, poate fi indusă excreția acestuia, prin permeabilizarea membranelor celulare (plasmalema şi tonoplastul). În acest sens a fost studiat efectul a numeroşi compuşi chimici asupra permeabilizării celor două membrane plasmatice

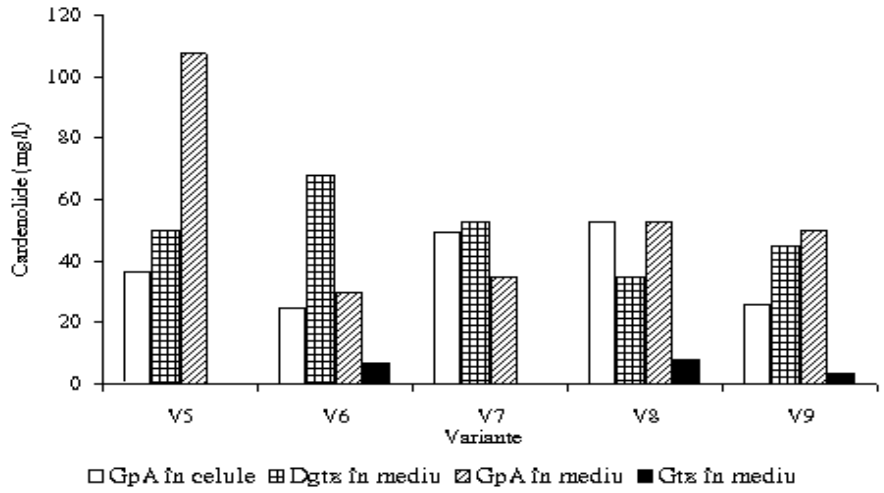
[6, 7, 13, 23]. De foarte multe ori s-a constatat însă că utilizarea unor astfel de compuşi are ca efect pierderea viabilităţii celulelor. Totuşi, la cele două linii celulare de *Digitalis lanata* luate de noi în studiu, aşa cum arătam mai sus, permeabilizarea membranelor celulare cu DMSO nu a fost asociată cu pierderea viabilităţii celulelor (Fig. 3A, 3B şi 4B).

Ceea ce trebuie însă remarcat cu deosebire este faptul că, alături de GpA, începând din ziua a 5-a, a fost excretată în mediu, dar numai de către linia celulară 15 (V1 şi V2), o altă cardenolidă, şi anume gitoxina (Fig. 5B3). Cantitatea acesteia este mică, fiind cuprinsă între 2,5 mg/l (V1) şi 3,5 mg/l (V2) şi a rezultat în urma hidroxilării dugitoxinei în poziţia C16, proces care a condus la biotransformarea digitoxinei în gitoxină. Această capacitate de biotransformare a digitoxinei în gitoxină, de către celulele de *Digitalis lanata* cultivate *in vitro* sub formă de suspensii sau imobilizate în alginat de calciu, nu a mai fost citată până în prezent decât în cazul culturilor de *D. purpurea* [16]. De asemenea, trebuie subliniat că acest glicozid cardiotonic, gitoxina, nu se acumulează în celule, el fiind excretat în totalitate în mediu.

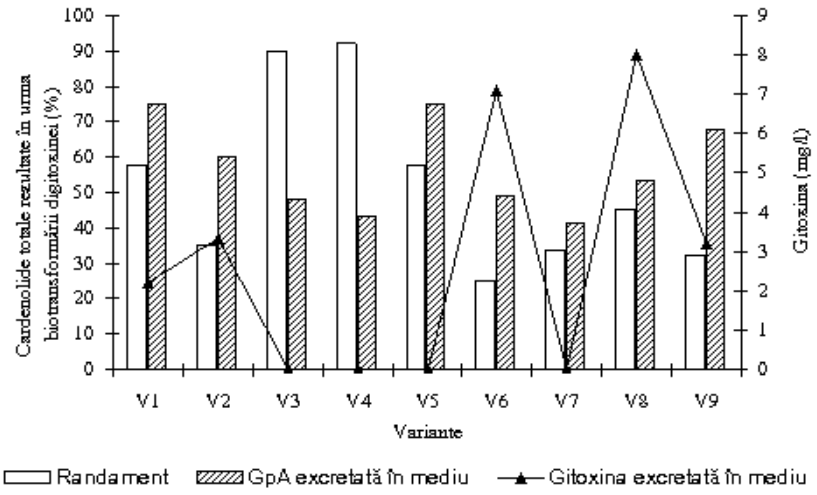
Introducerea digitoxinei dizolvată în DMSO, în doze mai mici (50 mg/l) şi succesive, din 3 în 3 zile (de 5 ori), nu a condus la mărirea randamentului de glucozilare a digitoxinei şi nici la cea a randamentului de hidroxilare, indiferent de linia celulară şi de compoziţia mediului folosit pentru biotransformare, ci a avut efect doar asupra cantităţii de GpA excretată în mediu, varianta V5 (linia 15) fiind cea mai prolifică în acest sens (Fig. 6). Cantitatea de digitoxină rămasă neutilizată se datorează, după părerea noastră, introducerii repetate şi la intervale scurte a unei noi cantităţi din acest compus, care nu a mai putut fi transformată de către celulele imobilizate.

Utilizarea metanolului ca solvent al digitoxinei, în condiţiile descrise mai sus, a avut ca rezultat inducerea unui proces asemănător de biotransformare a digitoxinei în gitoxină, ambele linii celulare (V6 şi V8) etalând valori aproximativ egale de bioconversie şi de eliminare a acestui compus în mediul lor de cultură (7 – 8 mg/l), dar mai mari decât în cazul utilizării DMSO (Fig. 6 şi 7). În acest sens, se pare că metanolul stimulează reacţia de hidroxilare a cardenolidelor, deoarece J o n e s şi V e l i k y [17], în experimentele de biotransformare efectuate cu *Daucus carota*, au observat că o concentraţie de metanol cuprinsă între 0,5 şi 1% a stimulat hidroxilarea digitoxigeninei, pe când la concentraţia de 2% a fost inhibată atât transformarea digitoxigeninei cât şi viabilitatea celulelor. Astfel că, şi în cazul nostru, este explicabilă producţia mică de compuşi rezultaţi în urma biotransformării, remarcată la variantele unde s-a utilizat metanolul în concentraţii de câte 0,5% per doză (V6 şi V8).

Dacă facem o comparaţie în ceea ce priveşte randamentul de biotransformare a digitoxinei în GpA, se observă că cel mai mare procent îl deţine în acest sens linia 78 (V3 şi V4), iar proporţia cea mai mare de cardenolide excretate în mediu (cca 75%), din total, s-a remarcat în cazul liniei 15 (V1 şi V5) (Fig. 7).



F i g. 6. *Efectul compoziţiei mediului de cultură şi al solventului digitoxinei asupra biotransformării digitoxinei de către celulele liniilor 15 şi 78 de* Digitalis lanata, *imobilizate în alginat de calciu*. GpA - Glicozida purpurea A. Dgtx – Digitoxină netransformată şi rămasă în mediu. Gtx – Gitoxina rezultată în urma biotransformării digitoxinei şi excretată în mediu. V5, V6, V7, V8, V9 - Variante (explicaţiile se găsesc în Tabelul 1).



F i g. 7. *Capacitatea de biotransformare a digitoxinei de către celulele celor două linii de* Digitalis lanata, *15 şi 78*, *imobilizate în alginat de calciu, în funcţie de compoziţia mediului de cultură, de momentul adăugării digitoxinei în mediu, precum şi de solventul utilizat*. GpA - Glicozida purpurea A. V1, V2, V3, V4, V5, V6, V7, V8, V9 - Variante (explicaţiile se găsesc în Tabelul 1).

Concluzii. 1. După mai multe selecţii s-a reuşit izolarea a două linii celulare de *Digitalis lanata* (dintre care una a fost hormon-habituată) care au prezentat proprietăţi deosebite în procesul de biotransformare a cardenolidelor. Imobilizate în alginat de calciu, aceste celule au fost capabile de a biotransforma digitoxina în glicozida purpurea A, într-o proporţie de cca 90%.

2. Utilizarea de DMSO (în concentraţia de 2%), pentru dizolvarea digitoxinei, a avut ca efect permeabilizarea membranelor celulare, fără pierderea viabilităţii celulelor imobilizate, astfel că o mare parte din cardenolidele acumulate în celule (între 45 şi 75%) a fost excretată în mediul de cultură.

3. Dintre cele două linii celulare, linia 78 (hormon-habituată) s-a dovedit a fi mai productivă şi mai rezistentă la acţiunea agenţilor solubilizanţi utilizaţi (DMSO şi metanol).

4. Ambele linii celulare selectate de noi şi imobilizate în alginat de calciu, în funcţie de solventul utilizat, precum şi de momentul şi cantitatea în care digitoxina a fost adăugată, au fost capabile şi de un proces de hidroxilare a acesteia în poziţia C16, proces în urma căruia rezultă gitoxina, care se acumulează numai în mediul de cultură, fapt constatat pentru prima dată în culturile celulare de *D. lanata*.

5. Metanolul a stimulat procesul de hidroxilare, dar a avut un efect puternic inhibitor asupra creşterii şi a viabilităţii celulelor celor două linii.

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ACCUMULATION OF SOME HEAVY METALS FROM MINE SPOILS BY SOYBEAN PLANTS

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SUMMARY. – The accumulation of Zn, Pb, Cu and Cd from mine spoils in Cavnic (Baia Mare mining area, Romania) by two soybean (*Glycine max*) cultivars, Agat and Diamant, was 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. The heavy metal concentrations in the cultivation substrata, as well as in soybean leaves and roots were measured. As compared to the control, the heavy metal concentrations in spoils increased as follows: Cd>Zn>Pb>Cu. The translocation of heavy metals (% of the total amount in the cultivation substrata) to soybean leaves followed the order: Zn>Cd>Cu>Pb. In all the experimental variants, the amount of Cu, Cd and Pb translocated to the leaves of the Agat and Diamant soybean cultivars ranged between the normal values, whereas Zn accumulation exceeded these values in both soybean cultivar plants grown in the variants with spoils. Reported to the total metal concentrations in the cultivation substrata and to the total amount of heavy metals absorbed by soybean plants, the percentage of the metals translocated to the leaves decreased, except for Pb in 100% spoils. With few exceptions, in all the experimental variants the Diamant cultivar plants accumulated higher amounts of heavy metals in their roots and leaves than did the Agat cultivar plants.

Generally, heavy metals represent a group of metals with density higher than 5 or 6 g/cm^3 [18, 28]. Zn, Cu, Fe, Mn and Mo, unlike Cd, Pb, Al, Hg, As, Cr, are required by biological systems as structural and catalytic components of proteins and enzymes, being essential to normal growth and development. A common characteristic of heavy metals in general, regardless of whether they are biologically essential or not, is that in excess they are strongly phytotoxic [17]. Although the relative toxicity of different metals to plants can vary with plant genotype and experimental conditions, the most phytotoxic metals, when present in excessive amounts, are Hg, Cu, Ni, Pb, Co and Cd [1].

Heavy metals occur naturally in rocks and soils, but mainly in forms that are not available to living organisms. Following the industrialisation, the most important source of heavy metals in the environment became the anthropogenic factor [27]. Mining activities represent one of such sources, resulting in huge amounts of spoils with negative consequences on the nearby ecosystems.

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The aim of the present study was to assess the chemical composition of the Pb-Zn-Cu mine spoils in Cavnic and the accumulation of some heavy metals in leaves and roots of the Agat and Diamant soybean cultivars. Of the heavy metals in the spoils, only those present in higher quantity (Zn, Pb, Cu, Cd) have been taken into consideration.

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 experimental plots (2-m^2 each) were organised into three variants:V_1 – control (unpolluted soil), V_2 – 50% spoils + 50% unpolluted soil, V_3 – 100% spoils. The spoils were collected from the old waste dump resulted from Pb-Zn-Cu ore processing in Cavnic, Baia Mare mining area, Romania; sampling depth was 0-50 cm. The unpolluted soil was a slightly degraded chernozem, with a pH of 6.9. The humidity of cultivation substrata has been maintained at about 70% of the water-holding capacity.

Samples of soil and spoils were taken from field plots. The samples were air-dried, ground and sieved through a 2-mm sieve prior to analyses. pH was measured at a 1:1 soil:water ratio using glass electrode and organic matter was measured by combustion. Total metals (Zn, Pb, Cu, Cd) in the soil and spoils (0.2 g) were extracted by digestion with a mixture of concentrated acids (10 ml HF, 2.5 ml HNO_3, 1 ml $HClO_4$). Zn and Cu concentration was determined by an inductively coupled plasma atomic emission spectrometer (ICP-AES, Jobin Yvon Sequential Spectrometer JY24), whereas Pb and Cd were determined by an inductively coupled plasma mass spectrometer (ICP-MS, VG Elemental Plasma Quad II). A replicate of the chemical analyses for soil and spoil Zn, Pb and Cu, with X-ray fluorescence spectrometer has been performed, the results being very close to those given by ICP-AES and ICP-MS. The X-ray fluorescence spectrometry has also been used for determining some micro- and macronutrients in soil and spoils.

Plant material for the analyses of heavy metals was harvested after the maturation period, washed with tap water and then with distilled water, oven-dried at 70^oC for 48 hours and ground in an agate mortar. Heavy metals (Zn, Pb, Cu, Cd) present in the leaves and roots of mature soybean plants (0.2 g of dried plant material) were extracted by digestion with 2 ml HNO_3 and 2 ml H_2O_2 and measured by ICP-AES.

Results and discussion. Some chemical properties of the cultivation substrata are given in Table 1.The pH of spoils is alkaline, leading to the binding and immobilisation of heavy metal ions and, consequently, to reducing of their bioavailability, many metals being retained very strongly by the soil components under alkaline and reducing conditions [27]. The higher amount of Ca in the spoils maintains an increased pH. The low contents of organic matter, P and K indicate the low fertility of the mine spoils.

Table 1

Some chemical properties of the cultivation substrata

Soil property (%, except for pH)	Experimental variants		
	Control	50% Spoils	100% Spoils
pH	6.99	7.69	8.24
Organic matter	10.61	6.89	3.34
SiO_2	64.04	71.15	79.05
Al_2O_3	13.53	10.30	7.18
Fe_2O_3	4.94	4.92	4.81
MnO	0.12	0.27	0.43
MgO	1.25	1.17	1.11
CaO	1.18	1.43	1.65
K_2O	2.09	1.95	1.83
P_2O_5	0.18	0.12	0.07

The total heavy metal contents in the cultivation substrata are presented in Table 2. As compared to the control, the heavy metal concentrations in spoils were much higher, the highest value being recorded for Cd (40 times higher) and the lowest one for Cu (only 3.5 times higher), while the contents of Zn and Pb were 15.7 and 13 times higher, respectively. In 50% spoils the heavy metal concentrations decreased to almost half of that in 100% spoils.

Table 2

Heavy metal contents of the soybean cultivation substrata

Experimental variants	Heavy metal concentrations (ppm)			
	Zn	Pb	Cu	Cd
Control	84.24	28.49	32.95	0.1953
50% Spoils	643.23	172.00	81.30	3.4520
100% Spoils	1325.55	379.35	116.26	7.8450

In Table 3 the heavy metal (Zn, Pb, Cu, Cd) contents in leaves and roots of the two soybean cultivars, Agat and Diamant, grown in different experimental variants, are given.

For both soybean cultivars and all studied metals (Zn, Pb, Cu, Cd), heavy metal concentrations in leaves and roots increased with their increasing levels in the cultivation substrata. The Cu amount in leaves of both soybean cultivars grown in the variants containing spoils was higher as compared to the control, but it was almost identical in the leaves of Diamant cultivar in 50% and 100% spoils (9.5 and 9.7 ppm, respectively), and the leaves of Agat cultivar in 100% spoils contained a lower amount of Cu (7.6 ppm) than those of plants grown in 50% spoils (8.9 ppm). In both soybean

cultivars and in all the experimental variants the roots had a higher concentration of heavy metals than the leaves. For all the cultivation variants and all the analysed heavy metals, less for Cu in control leaves and for Cd in roots in 50% spoils, the leaves and roots of Diamant cultivar plants contained a higher amount of heavy metals than those of Agat cultivar plants.

Table 3

Heavy metal contents in the leaves and roots of the Agat and Diamant soybean cultivars

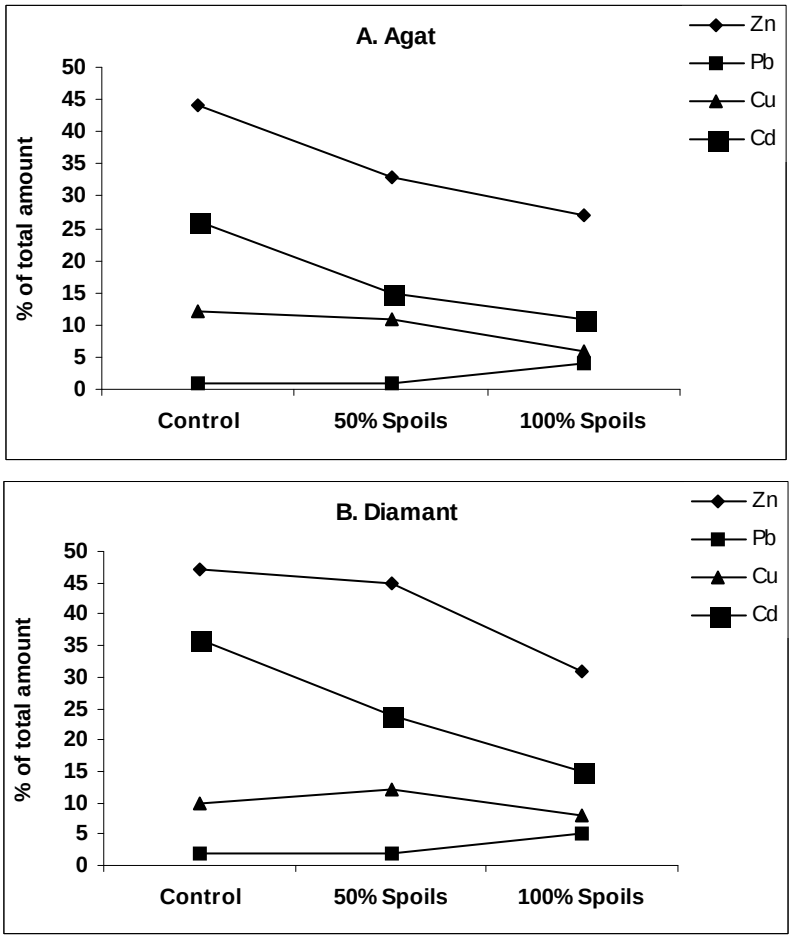
Cultivar	Experimental variants	Heavy metal concentrations (ppm)							
		Zn		Pb		Cu		Cd	
		L	R	L	R	L	R	L	R
Agat	Control	36.9	41.36	0.29	3.08	4.0	15.24	0.05	0.1158
	50% Spoils	212.7	264.60	1.38	20.43	8.9	22.50	0.53	2.3906
	100% Spoils	353.8	795.30	16.50	105.80	7.6	48.18	0.86	6.2347
Diamant	Control	39.5	43.74	0.55	4.75	3.2	15.36	0.07	0.1201
	50% Spoils	286.8	303.40	3.94	38.26	9.5	24.30	0.84	2.3220
	100% Spoils	417.7	819.40	18.60	128.40	9.7	51.72	1.17	6.2450

L - Leaves.
R - Roots.

Although the heavy metal levels in soybean leaves increased in plants grown in the variants containing spoils as compared to the control, still, when reported to the metal amounts in the cultivation substrata, the percentage of Zn, Cd and Cu translocated to the leaves of both soybean cultivar plants in 50% and 100% spoils was lower than in control (Fig.1, A, B). The percentage of translocated Cu slightly increased (12%) comparing to the control (10%) only in Diamant cultivar plants grown in 50% spoils.

Pb, although present in higher amount in spoils, was translocated in equal percentage to the leaves of both cultivar plants in control and 50% spoil variants and in a higher percentage in the plants grown in 100% spoils, but, of the four studied heavy metals, Pb was translocated to the leaves in the lowest percentage, in both cultivars. Thus, in Agat cultivar only 1% of the total amount of Pb in the cultivation substrata was translocated to the leaves of control and 50% spoil plants, and 4% in 100% spoil plants. In Diamant cultivar 2% of total Pb was translocated to the leaves of control and 50% spoil plants and 5% in 100% spoil plants. Also, just a small part of the total amount of Cu in the cultivation substrata was translocated to the leaves, in all the experimental variants and both soybean cultivars.

The total content of heavy metals in the soil (control) used in this experiment is in accordance with the “normal” levels for unpolluted soils, which range between the following values: Zn 10-300 (typical value 50) ppm, Pb 2-100 (10) ppm, Cu 2-100 (20) ppm, Cd 0.01-0.7 (0.06) ppm [5, 7, 13, 30].



F i g. 1. *The amount of heavy metals (% of the total amount in the cultivation substrata) translocated to leaves of the Agat şi Diamant soybean cultivars.*

The heavy metal contents in plants is influenced by a number of factors: the type of plant, the particular plant tissue, the levels and availability of metals in soil, the interactions between different elements in soil, the age of plants, the season, the climatic conditions. Because of these factors, the concentrations of the heavy metals vary widely, being hard to give adequate mean values [11]. However, the following "normal" values are indicated: Zn 25-150 ppm, Cu 5-20 ppm [4, 14], Pb 0.2-20 ppm, Cd 0.05-2 ppm [11].

Referred to the literature data, the amounts of Cu, Cd and Pb translocated to the leaves of Agat and Diamant soybean cultivars were within the limits considered "normal", whereas Zn accumulation exceeded these limits in both soybean cultivars in

variants with spoils (Table 3). According to some authors [6, 21, 22], the critical toxicity level (CTL) of Zn in the leaves of most crop plants is between 100-300 ppm; the CTL of Cu is above 20-30 ppm, and in the case of Cd it ranges between 3 and 10 ppm. M a c N i c o l and B e c k e t t [21] indicated a CTL of 4 ppm Cd in the mature leaves of soybean and a CTL of 450 ppm for Zn. B o r k e r t *et al*. [8] indicated a CTL of 140 ppm for Zn and over 20 ppm for Cu in soybean. The threshold concentration of Pb in plant tissues causing the first damage ranges from 500 to 1000 ppm, depending on the species [33].

Generally, most of the heavy metals taken up by plants accumulates in roots, being bound to the root cell walls [31, 33]. Plant roots often serve as storage sites preventing toxic dosages from reaching the stem and grain [12]. Zinc is known as the most mobile of the heavy metals [25], both in the soil and the plants [12]. Although Cd is generally present in soils in much smaller concentrations than Zn, it is also considered to be mobile, the two metals having similar chemical properties. As a result of high mobility, Zn and Cd are more available to plants than many other heavy metals, including Cu and Pb [2, 24, 27, 34]. C a t a l d o *et al*. [9] showed that although most of Cd taken up by soybean plants has been retained within the roots, an important fraction of the Cd associated with shoot tissues has been remobilised to seeds at maturity.

Studying the responses of different plant species to Cd, M a c L e a n [20] reported that Cd was present in higher concentrations in the roots than in other organs of soybean. Zinc is readily translocated to plant shoots, while copper accumulates mostly in roots, but none of these two elements moves appreciably into plant seeds [24]. A r d u i n i *et al*. [3], investigating Cd and Cu uptake and distribution in pine and ash, found that most of the Cu taken up by these plants accumulated in roots and only a small part was translocated to the leaves, whereas the percentage of translocated Cd was high. The roots restrict Cu translocation to the above-ground plant parts, due to the strong affinity of Cu for different binding sites on the cellulose discs of the cell walls [26]. Cu and Pb bind strongly to various soil components, mostly to organic matter, and tend to be the most rapidly adsorbed and the most slowly desorbed heavy metals [23, 27]. Most Pb accumulates in the root cell walls, which limit its translocation to the above-ground plant parts through the xylem [33].

The rate and extent of the movement of heavy metals within plants depend on the metal concerned, the plant organ and the age of the plant [1]. Within plants the metals may exist as free ions or as organic complexes, which influence their mobility [11]. Of the four studied metals, Zn and Cd were in the highest percentage translocated to the leaves of both soybean cultivars, followed by Cu and Pb (Fig. 1). As the amount of heavy metals in the cultivation substrata increased, the percentage of translocated metals increased, but reported to the total metal concentrations in

the cultivation substrata and to the total amount of heavy metals taken up by soybean plants, this percentage decreased, except for Pb (Fig. 1, Table 4). Similar results have been reported by other authors [3, 15, 29]. S a n t a-M a r i a and C o g l i-a t t i [29] explained their results for Zn by a higher accumulation of this metal into plant roots and its exclusion from the cytoplasm of root cells, and/or by pumping Zn into the vacuoles or by its complexation with organic compounds, thus restricting its translocation to the shoots and helping the survival and growth of plant species under toxic Zn levels. This mechanism seems not to work for soybean in our experiment, Zn being translocated to the leaves of plants grown in variants with spoils in concentrations exceeding normal values. Soil contamination with heavy metals seems to persist for long periods, but the mobility and bioavailability of heavy metals decrease over time [19].

Table 4

Heavy metal concentrations (% of the total amount taken up by plants) in leaves and roots of the Agat and Diamant soybean cultivars

Cultivar	Experimental variants	Heavy metal concentrations (%)							
		Zn		Pb		Cu		Cd	
		L	R	L	R	L	R	L	R
Agat	Control	47	53	9	91	21	79	30	70
	50% Spoils	45	55	6	94	28	72	18	82
	100% Spoils	31	69	13	87	14	86	12	88
Diamant	Control	47	53	10	90	17	83	37	63
	50% Spoils	49	51	9	91	28	72	27	73
	100% Spoils	34	66	13	87	16	84	16	84

L - Leaves.
R – Roots.

The decrease in the amount of Zn and Cd translocated to the soybean leaves could be caused by the high pH of spoils, the adsorption/desorption of Cd and Zn showing a greater sensitivity to pH than that of Pb and Cu [2]. Soils high in Fe, Mn and Al oxides preferentially retain Cu, Pb and, to a lesser extent, Zn [27]. Since the organic matter content was very low in mine spoils, the higher amount of aluminosilicates and MnO in the spoils could be one of the causes of reduced translocation of Pb and Cu to soybean leaves.

In broad terms, there is a positive relationship between the concentration of Pb in the soil and that in the plants, but only a small proportion of the lead in soil is available for uptake by plants [10]. Although the leaves of the Agat and Diamant soybean cultivars accumulated a high amount of Pb in 100% spoils, this Pb amount represented only 4 and 5%, respectively, of the total amount of Pb in spoils.

Plant cultivars differ widely in their ability to absorb, accumulate and tolerate heavy metals [2]. Leaves of the Diamant cultivar had a higher content of heavy metals than those of the Agat cultivar. W h i t e *et al*. [32] noticed that in soybean genotypes Zn tolerance was positively correlated with the Zn content of the leaves, therefore the mechanism of tolerance in this case was not exclusion from uptake, but tolerance of the tissues to high contents of Zn. K u b o i *et al*. [16] found that soybean plants were sensitive to Cd, but able to exclude it.

Conclusions. 1. Of the four heavy metals considered (Zn, Pb, Cu, Cd), the amount of Cu, Cd and Pb translocated to the leaves of the Agat and Diamant soybean cultivars, ranged between the "normal" values, whereas Zn accumulation exceeded these values in both soybean cultivar plants grown in the variants with spoils.

2. In all the experimental variants and for both soybean cultivars, most of the absorbed heavy metals accumulated in plant roots, while the percentage of metals translocated to the leaves was lower. As the amount of heavy metals in the cultivation substrata increased, the percentage of translocated metals increased, but reported to the total metal concentrations in the cultivation substrata and to the total amount of heavy metals taken up by soybean plants, this percentage decreased, except for Pb in 100% spoils.

3. The most mobile of the four studied heavy metals was Zn, followed by Cd, Cu and Pb.

4. Except for Cu in control leaves and Cd in the roots of plants grown in 50% spoils, in all the experimental variants the heavy metal contents in leaves and roots of the Diamant cultivar plants were higher as compared to the Agat cultivar plants.

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REGULATION OF THE EXPRESSION AND FUNCTIONS OF INTERENDOTHELIAL JUNCTIONAL PROTEINS IN CEREBRAL ENDOTHELIAL CELLS UNDER HYPOXIA AND OXIDATIVE STRESS

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SUMMARY. - Cerebral microvessels are implicated in the pathogenesis of several disorders of the central nervous system (CNS), such as neoplasia, ischemia, hypoxia, epilepsy, multiple sclerosis, dementia. The mechanism of the blood-brain barrier (BBB) disturbance following cerebral ischemia is largely unknown. In the increased permeability of the barrier, endothelial cells (ECs) and astrocytes are directly affected. In our study, we used the immortalised rat brain endothelial cell line GP8 to investigate the direct effect of hypoxia and reoxygenation on BBB-associated functions. For production of hypoxia (5% CO_2 and 95% N_2) and reoxygenation (5% CO_2, 70% N_2 and 25% O_2) we worked with a special chamber (Billups-Rothenburg). In addition, the oxidative stress was investigated by using 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), which continuously releases H_2O_2. We found significant changes in the expression of junctional proteins. While occludin and cadherin were downregulated in oxidative stress, no similar changes were seen in the case of ZO-2 and β-catenin. Our data indicate that changes in junctional protein-protein interactions may be involved in the damage of the barrier function caused by oxidative stress. We investigated the possible role of mitogen-activated protein kinase (MAPK) signal transduction pathway in the disruption of intercellular junctions. Our results suggest an activation of extracellular signal-regulated kinase (ERK1/2) pathway in response to DMNQ in GP8 cells. Protease activities (matrix metalloproteinases - MMPs and tissue plasminogen activator - tPA) also increased after DMNQ treatment. These two classes of neutral proteases act in concert in different neurological diseases which are associated with increased capillary permeability.

The blood-brain barrier (BBB) isolates brain cells from the normal variations in body fluid composition and regulates the composition of the brain's extracellular fluid, providing a stable environment for nerve cell function.

By forming a single cell layer, cerebral endothelial cells (CECs) constitute the principal component of the BBB. These cells are characterised by the presence of continuous tight junctions (TJs) and a low level of endocytosis/transcytosis.

The tight junction is one mode of cell-to-cell adhesion in epithelial and endothelial cells. They serve as a primary barrier preventing solutes and water from passing freely through the paracellular pathway ("barrier function"). TJs have also

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important role in the generation and maintenance of the cell polarity ("fence function"). Both the barrier and fence functions of TJs are essential for multicellular organisms [3].

The TJ proteins can be classified as:

1. integral membrane proteins: occludin, claudins, junction-associated membrane protein (JAM);

2. cytoplasmic plaque proteins, including the MAGUK (membrane–associated guanylate kinase homologue) proteins of TJ: zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2) and zonula occludens-3 (ZO-3) and a more heterogeneous group, including cingulin, symplekin, 19B1, AF-6 and 7H6 proteins; and

3. TJ-associated proteins, which are involved in signal transduction pathways: small GTPases-binding proteins (Rab3b, Rab13), two types of heteromeric G-proteins ($G\alpha_0$ and $G\alpha_{i2}$), atypical protein kinase C (PKC) isotypes.

The adherens junctions (AJs) mediate the initial adhesion between epithelial and endothelial cells and play a modulatory role. TJ formation in epithelial cells depends on the prior formation of E-cadherin-mediated AJs. Disruption of AJs is followed by the increase in TJ permeability. Thus, AJ proteins (cadherins, catenins, p120, p100) are potential regulators of TJs [11].

Materials and methods. *Cell cultures.* Primary cultures of rat brain endothelium were used to generate temperature sensitive SV40 large T immortalised cell line GP8 [4]. GP8 cells express P-glycoprotein, glutamate transporter-1 (GLUT-1), transferrin receptor, von Willebrand factor and the rat endothelial cell antigen-1 (RECA-1) and exhibit high affinity uptake of acetylated low density lipoprotein (LDL) and stain positive with the alpha-Gal specific binding lectin (BSI-B4). They also express major histocompatibility complex (MHC) class I and intercellular adhesion molecule-1 (ICAM-1) constitutively and can be induced to express MHC class II and vascular cell adhesion molecule-1 (VCAM-1) following cytokine activation. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is also expressed. GP8 cells retain many features of the CEC in primary cultures, and are considered as a useful tool for the study of BBB functions *in vitro*. GP8 cells (passages 40-50) were seeded on rat tail collagen-coated dishes and maintained as described earlier [6].

Treatments. Cerebral endothelial cells were subjected to hypoxia and reoxygenation *in vitro* in a special air tight chamber (Billups-Rothenburg). Hypoxia was performed in an atmosphere of 95% N_2 and 5% CO_2, while the subsequent reoxygenation in 70% N_2, 25% O_2 and 5% CO_2. Confluent monolayer of GP8 cells was subjected to oxidative stress either by using 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) or H_2O_2. DMNQ, a redox-cycling agent induces intracellular generation of reactive oxygen species, cell proliferation, apoptosis or necrosis in a concentration-dependent way. In our experiments DMNQ was used (1 μM and 10 μM) for 1.5 hours.

In other experiments 1 mM H_2O_2 was used in the presence of normal glucose concentration or under aglycemic conditions for 2-4 hours.

For phosphorylation studies, cells were subjected to 50 μM phenyl arsenic oxide (PAO), which is a tyrosine phosphatase inhibitor, for 1.5 hours [13].

For *Western blot analysis* protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane at 100 V constant voltage for 90 minutes. The membrane was preincubated in blocking buffer (5% non-fat dry milk, in Tris buffer saline/Tween 20 -TBS/T-: 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 30 minutes then incubated with primary antibody for 2 hours. After two washes for 15 minutes with TBS/T, the membrane was incubated with the horse radish peroxidase-conjugated secondary antibody for 1 hour at room temperature and washed again two times with TBS/T. The protein bands were detected using a chemiluminescent detection system.

For *immunoprecipitation assay* confluent monolayers of cells were lysed in 0.5 ml of appropriate lysis buffer and collected. After centrifugation (10,000 rpm, 5 minutes, 4°C), the supernatant was normalised for protein concentration and incubated with the primary antibody for 2 hours, followed by an additional 1 hour with protein A or protein G bound to Sepharose. After four washes in lysis buffer or ice-cold TBS/T, immune complexes were separated by adding SDS sample buffer followed by heating at 95°C for 3 minutes. Equal volumes of immunoprecipitated proteins and proteins from the supernatant were analysed by SDS-PAGE and immunoblotting.

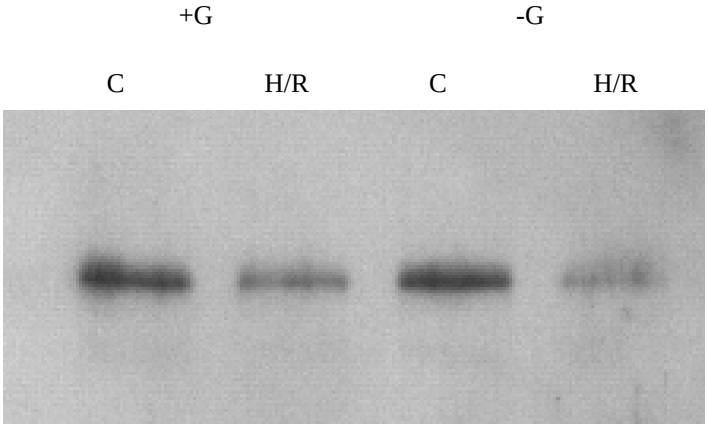
SDS–PAGE zymography. MMP enzyme activity was assayed by zymography: equal volumes of supernatant normalised for protein concentration (10 μl) were subjected to electrophoresis, without boiling or reduction, through a 10% polyacrylamide gel copolymerised with casein (0.5 mg/ml). After the electrophoresis was complete, the gel was incubated for 1 hour at room temperature in a 2.5% Triton-X100 and 50 mM Tris solution, then was washed two times, 10 minutes each, with distilled water and then incubated overnight at 37°C in a 20 mM Tris-HCl buffer, containing 100 mM $CaCl_2$. In parallel, the MMP activity in the culture media was studied. The gels were stained with 0.25% Coomassie blue R-250 and then destained with 10% methanol and 8% acetic acid. Enzyme activity attributed to MMPs can be visualised (on the basis of molecular weight) in the gelatin- or casein-containing zymograms as clear bands against a blue background [8].

To determine tissue plasminogen activator (tPA) activity, samples were electrophoresed through a 10% SDS-polyacrylamide gel. The gel was washed in 2.5% Triton and 50 mM Tris solution to remove the SDS, and in destilled water two times to remove the Triton-X100. The gel was placed on a semi-solid substrate containing 20 mM Tris-HCl, 1.5% agarose, 2% casein and 5 μg/ml plasminogen and incubated at 37°C overnight. tPA activity was visualised under dark-field illumination [9].

Results and discussion. The present work is an investigation of ischemic stress at the level of the cerebral capillary endothelium by analysing the changes in TJ and AJ protein expression, activation of signal transduction pathways and modification of the protease activity following hypoxia/reoxygenation and oxidative stress induced by DMNQ or H_2O_2.

1. Changes in the expression of junctional proteins after oxidative stress

Previous studies [1, 2] revealed that an *in vitro* model of the BBB is sensitive to short exposure to hypoxia/aglycemia and that changes in CEC calcium flux may be involved in the generation of the structural and functional variations of the BBB during ischemic stress. This study has shown that E-cadherin is decreased in a time-dependent manner after hypoxic/aglycemic exposures. Immunocytochemical studies revealed a change in the distribution of endothelial cell F-actin, too. These findings are consistent with our results indicating decrease in the expression of junctional proteins after oxidative stress (Fig. 1). Occludin is downregulated after hypoxia/reoxygenation, which is even more pronounced when the cells are cultured in a glucose-free medium. These changes are not accompanied by similar changes in ZO-2 expression.

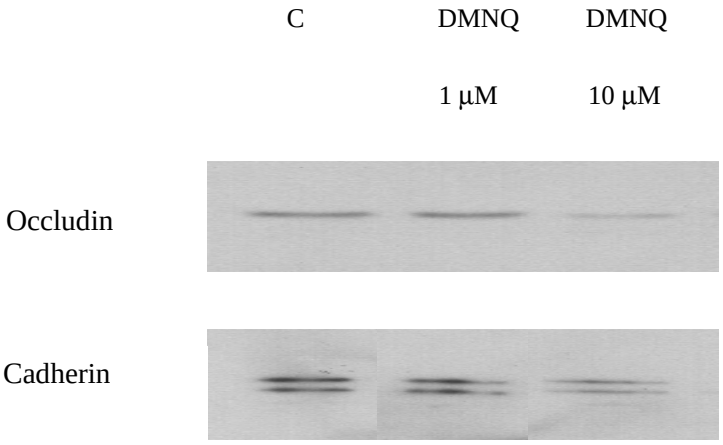


F i g. 1. *Expression of occludin after hypoxia/reoxygenation in GP8 cells.* C – Control. H/R - Hypoxia/reoxygenation. +G - Normal glucose concentration. -G - Aglycemic condition.

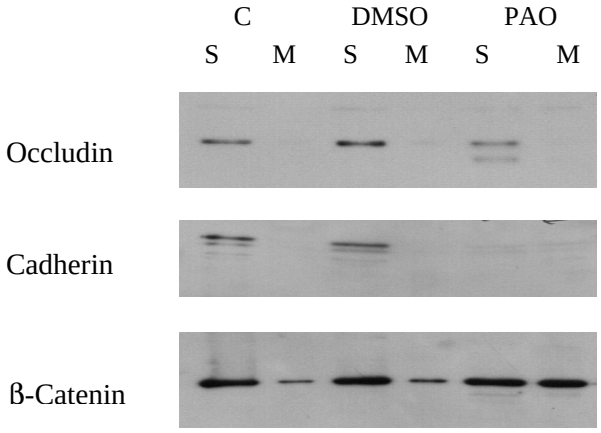
We found a dose-dependent reduction in the junctional protein expression after DMNQ treatment. Occludin and cadherin expression was reduced after DMNQ (10 μM) treatment (Fig. 2), while F-actin expression was not affected by DMNQ.

Previous studies [10] revealed an increase in the tyrosine phosphorylation of β-catenin after a tyrosine phosphatase inhibitor (PAO) treatment, which was associated with ZO-1 tyrosine phosphorylation. These findings indicate that BBB permeability could be regulated via mechanisms involving tyrosine phosphorylation

of junctional proteins. We found a reduction in the occludin, cadherin and ZO-2 expression after PAO treatment (Fig. 3). Reduction of occludin expression in human umbilical vein endothelial cells after PAO treatment was demonstrated [13]. We hypothesised that tyrosine phosphorylation of junctional proteins can be regarded as an essential step in the mechanism of intercellular junction disruption, followed by increase in BBB permeability after DMNQ treatment.

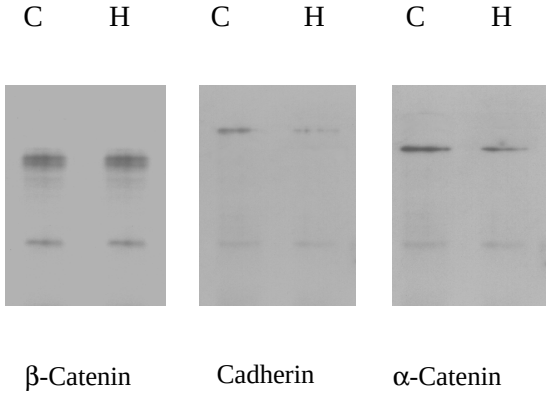


F i g. 2. *Expression of occludin and cadherin after DMNQ treatment in GP8 cells.*
C – Control.



F i g. 3. *Effect of tyrosine phosphatase inhibition on the expression of junctional proteins in GP8 cells.*
C - Control. DMSO - Solvent (dimethyl sulphoxide) control.
PAO (phenyl arsenic oxide) - 50 μM. S - Supernatant. M - Membrane fraction.

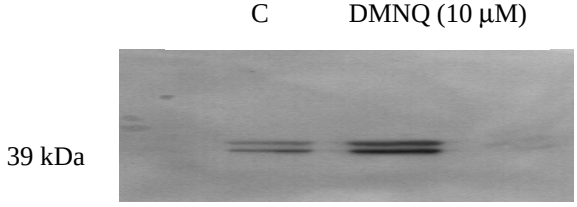
Immunoprecipitation assay indicated a reduction in the cadherin and α-catenin expression by oxidative stress, which was not accompanied by the reduction of β-catenin expression after H_2O_2 treatment (Fig. 4). Our data suggest an alteration in the protein-protein interaction of AJs during oxidative stress.



F i g. 4. *Interaction of AJ proteins after H_2O_2 treatment in cerebral ECs.*
C – Control. H - Hypoxia.

2. MAPK activation after DMNQ treatment

Recently, studies have shown that hypoxia induces endothelial dysfunction in bovine aortic endothelial cells through PKCα-mediated Ras/Raf-1/ERK1/2 pathway [7]. We hypothesised that in DMNQ-induced oxidative stress the mechanism of the damage of ECs involves the mitogen-activated protein kinase (MAPK) signalling pathway. Western blot analysis of extracellular signal-regulated kinases (ERK1/2, also termed p44 and p42) was performed with an antibody recognising only the catalytically activated enzymes (phosphorylated at Thr202/Tyr204). We found an increased ERK1/2 expression after DMNQ treatment (Fig. 5), which supports the hypothesis that MAPKs are involved in the mechanism of the BBB damage induced by oxidative stress.

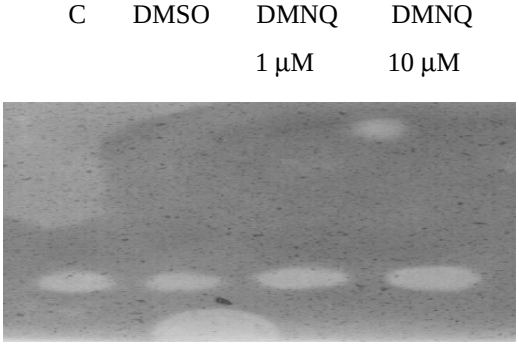


F i g. 5**.** *Activation of MAPKs (ERK1/2) induced by DMNQ treatment.*
C – Control.

3. Protease activities after DMNQ treatment

Matrix metalloproteinases (MMPs) are associated with the opening of the BBB, but their cellular localisation and activation mechanism are not completely understood. Gelatinase A (MMP-2) and gelatinase B (MMP-9) are able to digest the endothelial basal lamina, which plays a major role in maintaining BBB impermeability. Previous studies have shown that the early activation of MMP-2 and MMP-9 is associated with the early formation of vasogenic edema after focal cerebral ischemia in mice. MMPs produced by human cerebral endothelial cells (HCEC) are actively involved in the shedding of soluble adhesion molecules at the BBB [5]. Our data show an increase in the MMP-2 activity in GP8 cells after DMNQ treatment. This increase in MMP-2 activity is time- and concentration-dependent. We suggest that MMPs of the CECs could be directly engaged in the proteolytic disruption of intercellular junctions of BBB after ischemia.

Plasminogen activators are neutral proteases which act in concert with MMPs to attack the extracellular matrix. Tissue plasminogen activator (tPA), a serin protease, converts plasminogen into active plasmine. It is thought to function primarily in the fibrinolytic pathway. In CECs, expression of tPA is regulated by astrocytes via transforming growth factor-β [12] and its activity increases after inflammation. We found a concentration-dependent increase in tPA activity in CECs after DMNQ treatment (Fig. 6).



F i g. 6. *tPA activation after DMNQ treatment in GP8 cells.*
C – Control. DMSO - Solvent (dimethyl sulphoxide) control.

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EFFECT OF NICKEL ON WHEAT PLANTS, SOIL MICROORGANISMS AND ENZYMES

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SUMMARY.- In a pot experiment, 21-day-old wheat plants were treated with $NiCl_2$ at rates of 0, 0.02, 2 and 200 mg Ni/kg soil. On day 14 after this treatment, plants and soil were sampled and analysed for evaluation of the effect of Ni on the plant growth, chlorophyll content, respiration and transpiration intensity, on the numbers of some soil microorganisms (aerobic heterotrophic, ammonifying, aerobic free-living N_2-fixing bacteria and, separately, *Azotobacter* cells, as well as actinomycetes and micromycetes) and on the soil dehydrogenase, protease, endoglucanase, cellobiohydrolase and β-glucosidase activities.

The results have shown that plant growth was stimulated by the low rate of Ni and inhibited by the high Ni rate. Chlorophyll content decreased, while intensity of respiration increased at each Ni rate. Transpiration was intensified by the low and medium Ni rates and reduced by the high Ni rate. Nickel strongly reduced the numbers of soil microorganisms, excepting the number of *Azotobacter* cells, which exhibited a significant increase at the low Ni rate and insignificant increases at the medium and high Ni rates. The enzyme activities were very sensitively affected by the medium and high Ni rates.

In continuation of the investigations of our research group on environmental pollution by heavy metals (*e.g.*[7,8,11-15]), we have carried out a pot experiment for studying the effect of nickel added to soil at different rates on the growth and some physiological parameters of young wheat plants and on the numbers of some microbial groups and activities of some enzymes in the rhizosphere soil.

Materials and methods. A neutral soil (pH in H_2O 7.1), containing 2.09% $CaCO_3$, 2.5% humus, 0.2% total N, 9.8 mg P_2O_5 and 35.5 mg K_2O/100 g, was used. The soil placed into Mitscherlich pots was sown with wheat (variety Balkan). The pots were kept in greenhouse at 25°C and 16-hour photoperiod. After 21 days of plant growth, the soil was treated with a $NiCl_2$ solution at rates of 0, 0.02, 2 and 200 mg Ni/kg soil. For analyses, the plants and soil were sampled 14 days after addition of Ni.

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Plant growth wan estimated by determining dry matter biomass of roots and above-ground plant parts. The physiological parameters studied were: chlorophyll (a+b) content determined spectrophotometrically after extraction with 80% acetone according to the method of Arnon [1]; respiration measured manometrically in Warburg apparatus and transpiration estimated gravimetrically.

The numers of the following physiological and taxonomical groups of soil microorganisms were determined: aerobic heterotrophic bacteria; ammonifying bacteria; aerobic free-living N_2-fixing bacteria and, separately, *Azotobacter*, as well as actinomycetes and micromycetes. The nutrient media recommended in [4,16] were used.

Five soil enzyme activities, namely dehydrogenase and protease activities and those of three enzymes belonging to the cellulose-hydrolysing enzyme complex (endoglucanase, cellobiohydrolase and β-glucosidase) were measured. Reduction of 2,3,5-triphenyltetrazolium chloride served for determination of dehydrogenase activity according to Thalmann [18]. Gelatin was used as substrate for determination of protease activity [17]. The substrates used for determination of endoglucanase and cellobiohydrolase activities were carboxymethylcellulose and microcrystalline cellulose, respectively [9]. *p*-Nitrophenyl- β-glucoside served as substrate for determination of β-glucosidase activity [6].

All analytical data were submitted to statistical evaluation.

Results and discussion. *Effect of nickel on wheat plants.* Table 1 shows that the effect of Ni^{2+} on plant growth (formation of biomass) was depedent on its rate. In comparison with the untreated control, dry biomass of both root and above-ground part was increased by the low Ni rate (0.02 mg Ni/kg soil). But the increase was statistically insignificant. At the medium Ni rate (2 mg Ni/kg soil), dry biomass of root decreased insignificantly and that of above-ground part increased insignificantly. The high Ni rate (200 mg Ni/kg soil) caused a significant reduction of the dry biomass of both root and above-ground part.

Table 1

Effect of Ni^{2+} on plant growth (formation of biomass)

Ni addition (mg/kg soil)	Dry matter (mg/plant)	
	Root	Above-ground part
0 (control)	38.2	263.0
0.02	41.0	289.2
2	36.7	265.6
200	27.9	123.6
LSD 5%	4.9	33.2

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Table 2

Effect of Ni^{2+} on chlorophyll content and intensity of respiration and transpiration

Ni addition (mg/kg soil)	Chlorophyll content (mg/g d.m.*)	Respiration intensity (μl O_2/g d.m.)		Transpiration intensity (g H_2O/g d.m)
		Root	Above-ground part	
0 (control)	11.8	168.0	29.0	1.40
0.02	10.7	192.0	37.0	1.90
2	9.5	255.0	51.0	1.60
200	9.3	211.0	39.0	1.20
LSD 5%	1.1	21.6	4.1	0.15

*d.m. - dry matter.

The effect of Ni^{2+} on the physiological parameters studied was also dependent on its rate (Table 2). The chlorophyll content was reduced significantly by the low Ni rate and more significantly by the medium and high Ni rates. Respiration of both root and above-ground part was stimulated by each Ni rate; the stimulation was highest at the medium Ni rate. Transpiration increased significantly at the low and medium Ni rates and decreased significantly at the high Ni rate.

Effect of nickel on soil microorganisms. It is evident from Table 3 that the numbers of aerobic heterotrophic bacteria, ammonifying bacteria, actinomycetes and micromycetes were decreased very significantly by each Ni rate. There was a parallelism between the decreasing effect of Ni on the numbers of aerobic heterotrophic bacteria, actinomycetes and micromycetes and its rate. Contrarily, the decreasing effect on the number of ammonifying bacteria became smaller with increasing Ni rate.

Table 3

Effect of Ni^{2+} on the numbers of soil microorganisms*

Ni addition (mg/kg soil)	A.h.b. (x 10^6)	A.b. (x 10^5)	A.f.l.N.f.b (x 10^5)	A.c. (x 10^2)	Am (x 10^5)	Mm (x 10^4)
0 (control)	261.03	139.51	202.27	16.31	24.00	22.09
0.02	63.41	23.56	209.47	29.54	7.97	1.53
2	54.34	25.97	157.00	19.02	6.07	1.45
200	47.74	34.32	103.33	20.08	5.89	1.02
LSD 5%	14.17	3.09	10.41	5.08	1.55	0.65

*The numbers are reported to 1 g of dry soil.
A.h.b. - Aerobic heterotrophic bacteria. A.b. - Ammonifying bacteria. A.f.l.N.f.b. - Aerobic free-living N_2-fixing bacteria. Ac - *Azotobacter* cells. Am - Actinomycetes. Mm - Micromycetes.

The number of the aerobic free-living N_2-fixing bacteria was insignificantly increased by the low Ni rate and significantly decreased by the medium and high Ni rates.

Each Ni rate resulted in increased number of *Azotobacter* cells; the increase was significant at the low Ni rate and insignificant at the medium and high Ni rates. This finding is in good agreement with some literature data, according to which *Azotobacter* manifests tolerance for metallic and non-metallic ions [2]. The tolerance for Ni ions may be attributed to their sequestering by polyphosphate-containing cytoplasmic inclusions (volutin granules) [5] and to their chelating by free histidine molecules [10].

Effect ot nickel on soil enzymes. One can deduce from Table 4 that Ni at its medium and high rates significantly inhibited each enzyme activity. The low Ni rate had an insignificant stimulating effect on dehydrogenase activity and significant inhibiting effects on the other enzyme activities. The negative effect of Ni on each enzyme activity increased with its increasing rate.

Table 4

Effect of Ni^{2+} on soil enzyme activities*

Ni addition (mg/kg soil)	Dehydro-genase	Protease	Endoglu-canase	Cellobio-hydrolase	β-Glucosidase
0(control)	379.00	16.25	0.278	0.054	0.182
0.02	390.00	14.25	0.200	0.035	0.146
2	256.00	14.00	0.153	0.020	0.114
200	200.00	12.07	0.104	0.005	0.095
LSD 5%	28.52	1.28	0.006	0.003	0.008

* The activities are reported to 1 g of dry soil and expressed in μg triphenylformazan (dehydrogenase), gelatin units (protease), mg reducing sugars (endoglucanase and cellobiohydrolase) and μmol *p*-nitrophenol (β-glucosidase).

Comparison of the analytical data in Tables 1-4 with each other makes it evident that the soil enzyme activities responded more sensitively to the medium and high (polluting) Ni rates than did the wheat plants and soil microorganisms. This finding supports the view of Dick [3] who considers soil enzyme activities as integrative indicators of soil health.

Conclusions. 1. Plant growth (formation of root and above-ground plant biomass) was stimulated by the low rate of Ni and inhibited by the high Ni rate.

2. Chlorophyll content decreased, while intensity of respiration of both root and above-ground plant part increased at each Ni rate. Transpiration was intensified by the low and medium Ni rates and reduced by the high Ni rate.

3. Nickel strongly reduced the numbers of soil microorganisms, excepting the number of *Azotobacter* cells, which exihibited a significant increase at the low Ni rate and insignificant increases at the medium and high Ni rates.

4. The enzyme activities were very sensitively affected by the medium and high Ni rates.

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SOIL ENZYMOLOGICAL METHODS ELABORATED TO BE USED FOR DETECTING EXTANT AND/OR EXTINCT LIFE ON THE PLANET MARS

STEFAN KISS•

SUMMARY.- This article is a brief review of the soil enzymological investigations carried out with the aim to elaborate methods applicable for detecting extant and/or extinct life on other planets, notably Mars. As the results of the experiments performed with the two Viking landers set down on Mars suggest that Mars is, today at least, a lifeless planet, search for extinct life on Mars by using soil enzymological methods appears to be an attractive objective for exobiological research.

Introduction. Enzyme activity in soil results from the enzymatic activity of living cells and from the activity of accumulated enzymes. A part of the accumulated enzymes are present in dead cells and survive, remain active for long time after the death of cells [5]. Therefore, the soil enzymological methods make it possible to measure both extant and extinct biological activity [1].

The enzymatic life detection on Mars should comprise the steps specified below. The spacecraft settled onto the surface of Mars takes samples from the Martian soil and introduces them into the reaction chamber containing the solution of enzyme substrate. The enzyme reaction is then followed on Earth by remote sensing of one of the reaction products [1, 2, 10, 11].

For elaboration of the soil enzymological methods to detect life on Mars mostly desert soils were used to simulate the Martian environment. These methods should be very sensitive, requiring small quantities of soil and short incubation times. With these methods, even trace amounts of reaction product should be accurately measurable and easily detectable by remote sensing.

The first investigations for elaboration of soil enzymological methods to be used in exobiological research were carried out by H o c h s t e i n [2] under a grant sponsored by the National Aeronautics and Space Administration (NASA). As described in his report, fluorometric methods were elaborated for determination of soil phosphatase and peptidase activities. α-Naphthylphosphate and L-leucyl-β-naphthylamide, respectively, served as enzyme substrates. They are not fluorescent, but their hydrolytic products, α-naphthol and β-naphthylamine, respectively,

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fluoresce. Intensity of their fluorescence is proportionate to the level of phosphatase and peptidase activities, respectively. The activities were detectable after short incubation times (1-4 hours) and required small quantities of soil (10-100 mg soil per ml of reaction mixture). The number of samples collected from different California soils was 59 for phosphatase activity and 35 for peptidase activity. The conclusion was drawn that the method for measuring peptidase activity appears to be a better candidate for life detection than is the phosphatase method.

It should be mentioned here that R a m í r e z- M a r t í n e z and M c L a r e n [7], referring to the method of H o c h s t e i n [2], used Na-β-naphthylphosphate and Na-β-glycerophosphate for determination of phosphatase activity in three California soils. The fluorometric method was found to be more sensitive and, thus, more rapid than the method with glycerophosphate.

Further investigations. Under a contract sponsored by NASA, W e e t a l l *et al*. [11] (Space Sciences Division, Jet Propulsion Laboratory, California Institute of Technology, Pasadena) elaborated a method for determination of soil catalase activity. Samples of several desert soils and of a garden soil were studied. The soil samples were treated with a solution of lysozyme which hydrolyses the cell wall of many, but not of all, bacteria and, consequently, the cell-bound catalase molecules are released from the lysed cells. After the lysozyme treatment, a hydrogen peroxide (H_2O_2) solution is added to the soil suspension and the molecular oxygen resulted from the catalase-catalysed decomposition of H_2O_2 is estimated manometrically. The catalase method elaborated can detect 10^3-10^4 microbial cells in a relatively short time, and it is supposed that with an improved technique for lysing microorganisms and a more sensitive method for measuring O_2, it may even be possible to detect 1-10 lysed microbial cells.

Detection of life by urease activity was also suggested [1]. A solution of ^{14}C-labelled urea is sprayed onto a sample of Martian soil, and then evolution of radioactive carbon dioxide is monitored. The decision to choose urease, in preference to other enzymes, is based on the assumption that the substrate urea may have arisen through abiological synthesis and, thus, any primitive living system may possess enzymes capable to use it as carbon and nitrogen source.

The dry valleys of South Victoria Land, Antarctica form the coldest and driest desert region of the Earth and were studied microbiologically as a model environment for investigating questions connected with the biological exploration of Mars [3]. Although enzyme activities in soils of these valleys were not measured, some microbiological findings, namely the presence of metabolic activity ($^{14}CO_2$ production from added labelled glucose and amino acids) even in soils lacking viable (culturable) microorganisms, were attributed to survival of active enzymes in the dead microbial cells.

It is noteworthy that, although a biodeteriorated stone monument can, of course, not be regarded as a model of Martian soil, our research group [4, 6] has found that detection of life in such a stone can easily be carried out through estimation of invertase activity in a small quantity of powered stone, by a simple paper chromatographic technique.

A hope – Martian soil samples will be analysed enzymologically on the Earth. S h i h and S o u z a [9] (Extraterrestrial Biology Division, Ames Research Center, NASA, Moffett Field, California) elaborated soil enzymological methods to be used not on Mars, but in the situation briefly characterised below. The spacecraft will bring soil samples of Mars to the Earth. Such samples must be treated as potentially hazardous materials and, therefore, they must be sterilised. The sterilising agent (dry heat or γ-irradiation) should maximise the destruction of microorganisms and minimise the loss of enzymatic activities (biochemical information), allowing thus to detect life in the Martian soil samples by enzyme analyses performed on the Earth. Samples of an acid California soil of sandy loam texture were used. Urease, lactate decarboxylase and phosphatase activities were determined. ^{14}C-Urea, ^{14}C-lactate and β-naphthylphosphate were the substrates. For urease and decarboxylase activities the released $^{14}CO_2$ and for phophatase the released β-naphthol were measured. The results have shown that combining sublethal doses of dry heat and γ-irradiation effectively sterilised the soil and yielded enzymatic activities higher than those of soil sterilised by dry heat alone, but lower than those of soil sterilised by γ-irradiation.

Conclusion. According to S a g a n [8], results of the three experiments performed with the two Viking landers set down on Mars by NASA in 1976 suggest that "Mars is, today at least, a lifeless planet". Based on this statement one can draw the conclusion that search for extinct life on Mars by using soil enzymological methods appears to be an attractive objective for exobiological research.

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STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, XLVII, 1, 2002

BIBLIOGRAPHY OF ENVIRONMENTAL ENZYMOLOGY IN ROMANIA. III

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The first and second Bibliographies of Environmental Enzymology in Romania were published in the volume "Fifth Symposium on Soil Biology (Iaşi, 1981)" (Romanian National Society of Soil Science, Bucharest, 1984, pp. 183-207) and in the journal "Studia Universitatis Babeş-Bolyai, Biologia" (1996, **41** (1-2), 217-235). The papers appeared after 1996 and those not included in Bibliographies I and II are listed in the present Bibliography.

The papers in Bibliographies I and II were grouped into 8 chapters, but Bibliography III comprises only Chapters 1-4 and 8, as Chapters 5 (Compost and farmyard-manure enzymology), 6 (Humus enzymology) and 7 (Clay enzymology) are not represented.

The papers in Bibliography III are numbered in continuation of the last paper in Bibliography II (paper 394).

In Bibliography III, as in Bibliographies I and II, diploma theses, doctoral dissertations, abstracts and summaries of papers, unpublished contractors' reports and papers under press are not included.

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3. ENZYMOLOGY OF NATURAL WATERS AND BOTTOM SEDIMENTS, INCLUDING THERAPEUTIC MUDS

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8. MISCELLANEA

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RECENZII - BOOK REVIEWS

Wolfgang Burghardt and Christine Dornauf (Editors), **First International Conference on Soils of Urban, Industrial, Traffic and Mining Areas. Proceedings,** Universität-GH Essen, Germany, 2000, XXVIII +1098 pages with 340 tables and 272 figures in the test.

The First International Conference on "Soils of Urban, Industrial, Traffic and Mining Areas" (SUITMA) was held in Essen, on 12-18 July 2000. Its Proceeedings were published in three volumes which are structured into Introduction (Author index, Table of contents, Preface), 16 chapters, an addendum (City posters and late contributions) and Subject index, and contain 198 papers elaborated by 380 authors from 37 countries representing Europe, North and South Americas, Asia, Africa, Australia and New Zealand.

The Preface was written by the Chairman of the Working Group SUITMA of the International Union of Soil Sciences, the distinguished German soil scientist, Professor Wolfgang Burghardt (University of Essen), who emphasises that the First International Conference on SUITMA brought numerous soil scientists together with the aim to present the available knowledge and to start a debate on what of soil information is essential for the sustainable development of cities. It is also stated in the Preface that the authors presented research work, results, information and problems on SUITMA on a world-wide dimension, but the themes of the conference were selected from the experiences made in Europe, where the western countries are mainly interested in urban pollution problems, while the eastern countries are much more engaged in questions of soil microbiology and soil hygiene of urban areas.

Volume I is entitled "*The Unknown Urban Soil - Detection, Resources and Faces*" and consists of 8 chapters: 1. City management and soils (no paper); 2. Soil heritage in urban areas (4 papers); 3. Reports on soils observed at urban sites (10 papers); 4. Methods and examples of field surveys (19 papers); 5. Analytical methods and their applicability (5 papers); 6. Classification - soil substrate, soil development, soil use (16 papers); 7. Relationships of soil use and history to soil features and development (4 papers); 8. Man-made materials, their features and quality as soils (9 papers).

Volume II, "*Application of Soil Information*", comprises chapter 9 with 7 subchapters; 9.1. Communal and industrial sludges and wastes (8 papers); 9.2. Storm water infiltration, ground water recharge, drainage, irrigation (11 papers); 9.3. Vegetable garden areas, food supply (5 papers); 9.4. Green areas, park and urban forest areas (5 papers); 9.5. Playground and sports field (1 paper); 9.6. Burial ground (no paper); 9.7. City planning (7 papers).

Volume III, "*The Soil Quality and Problems: What Shall We Do?*", contains chapters 10-16. Their themes are the following: 10. Quality of urban soils (16 papers); 11. Soil protection and soils as biotopes/pedotopes (5 papers); 12. Soil degradation (chemical, physical, biologi-

cal) (12 papers); 13. Soil remediation (14 papers); 14. Specific problems of industrial sites (14 papers); 15. Specific problems of traffic sites (sealing, de-icing, wetting, emissions/road, railway, airport, harbour) (10 papers); 16. Specific problems of mining sites (19 papers).

The addendum (City posters and late contributions) includes 4 papers.

All the papers published in the three volumes of the Essen Conference have contributed to a better knowledge of the SUITMA and have shown that the quality, degradation and functions of the SUITMA are wide-spread modern and global themes. Therefore, these volumes are fundamental sources of information for soil and other environmental scientists and also for the authorities and others responsible for the quality of life in urban, industrial, traffic and mining areas.

DANIELA PAŞCA and
STEFAN KISS

N.A. Kireeva, V.V. Vodop'yanov, A.M. Miftakhova, **Biologicheskaya aktivnost' neftezagryaznennykh pochv** (*Biological Activity of Oil-Contaminated Soils*), Izdatel'stvo (Publishing House) Gilem, Ufa, Bashkiria (Bashkortostan), Russian Federation, 2001, 377 pages with 138 tables and 118 figures in the text.

This book is a monograph devoted to summarising description of the long-term (more than 10-year) field and laboratory investigations conducted by Professor N.A. Kireeva (Bashkirian State University, Ufa) on soils contaminated with crude oil and oil products in Bashkiria, one of the oldest large centres of extraction and processing of mineral oil.

The book consists of Introduction, List of abbreviations, four chapters, List of references and Table of contents.

Complexity of the investigations described in the book is very evident even from the chapter and subchapter titles as specified below.

Chapter I. *Some Indicators of the Biological Activity of Soils Contaminated with Crude Oil and Oil Hydrocarbons*; 1.1. Dynamics of the number and activity of microorganisms; 1.2. Hydrocarbon-oxidising microorganisms; 1.3. Dehydrogenase and catalase activities; 1.4. Phenol oxidase; 1.5. Ascorbate oxidase; 1.6. Intensity of soil respiration; 1.7. Actinomycetes; 1.8. Aerobic spore-forming bacteria; 1.9. Microorganisms of the N cycle; 1.10. Enzymes of the N metabolism; 1.11. Cellulose-degrading microorganisms. Intensity of cellusose degradation. Mathematical model; 1.12. Activity of carbohydrases; 1.13. Phosphohydrolases; 1.14. Enzymes of the S metabolism; 1.15. Kinetics of the enzyme reactions; 1.16. Choosing of the biodiagnostic parameters as indicators of oil-contaminated soils; 1.17. Mathematical models of the dynamics of changes in numbers of microorganisms in polluted soil. Primary models for oil-contaminated soil; 1.18. Mathematical model of the oil degradation in sterile and natural soil; 1.19. Changes in soil properties and the degradation of oil; 1.20. Conclusion.

Chapter 2. *Microbiota of Soils Contaminated with Hydrocarbons*; 2.1. Yeasts and yeast-like fungi; 2.2. Micromycetes: numbers (colony-forming units), length of mycelia, biomass; 2.3. Species composing the community of micromycetes; 2.4. Cellulose-hydrolysing activity of fungi; 2.5. Radial growth rate of the colonies of soil micromycetes; 2.6. Hydrocarbon-utilising

activity of micromycetes; 2.7. Phytotoxicity of micromycetes; 2.8. Conclusion.

Chapter 3. *Phytotoxicity of Soils Contaminated with Crude Oil and Oil Products, and Growth of Plants on Oil-Contaminated Soils*; 3.1. Toxicity of soil; 3.2. Productivity of oats; 3.3. Productivity of barley, vetch and vetch-barley mixture; 3.4. Productivity of sweetclover; 3.5. Growth and development of weeds; 3.6. Conclusion.

Chapter 4. *Biological Activity of Recultivated Soils;* 4.1. Approaches to recultivation of oil-contaminated soils; 4.2. Application of mineral, organic and combined fertilisers; 4.3. Intensification of biodegradation of oil hydrocarbons by green manures; 4.4. Utilisation of agricultural wastes; 4.5. Application of biohumus; 4.6. Application of surface-active substances; 4.7. Utilisation of hydrocarbon-oxidising microorganisms for degradation of crude oil and oil products in soil; 4.8. Isolation of hydrocarbon-oxidising microorganisms, study of the conditions of their utilisation for destruction of oil in soil; 4.9. Production of a biopreparation with hydrocarbon-oxidising microorganisms and its application for recultivation of oil-contaminated soils; 4.10. Mathematical modelling of the process of oil biodegradation in sterile and natural soil after introduction of hydrocarbon-oxidising microorganisms; 4.11. Utilisation of activated sludge containing hydrocarbon-oxidising yeasts; 4.12. Possibility of the utilisation of a protein-vitamin concentrate for recultivation of oil-contaminated soils; 4.13. Conclusion.

The book has 749 references, of which 470 are in Russian and 279 in other languages.

Biological Activity of Oil-Contaminated Soils is a very useful source of information for a broad circle of experts: soil, plant and enviromental scientists and technologists and also for decision makers in taking measures for enviromental protection and rehabilitation of oil-contaminated soils.

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