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Microbial activity in the subterranean environment of Dârninii Cave, Bihor Mountains

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Abstract. Six cave material samples from Dârninii Cave, Bihor Mountains, were microbiologically and enzymologically analyzed. The microbiological analyses consisted in determining the presence of oligotrophs, aerobic heterotrophs, ammonifiers, nitrate and nitrite bacteria, denitrifiers and determining the presence of micromycetes. In order to form a complete image on the microbial processes from this habitat, the bacteriological analyses were completed with quantitative and qualitative enzymological analyses. At all the six samples of cave material the next quantitative enzymatic activities were performed: actual and potential dehydrogenase, catalase and phosphatase. Only regarding the intensity of the processes differences were noticed. The qualitative enzymatic activities: amylase, dextranase, saccharase, maltase and levanase, were not present at studied samples.
Keywords: microbiological analyses, enzymological analyses, Dârninii Cave.

Zusammenfassung. Sechs Materialproben aus der Dârninii Höhle in den Bergen von Bihor wurden mikrobiologisch und enzymologisch analysiert. Die mikrobiologischen Analysen bestehen aus dem Nachweis von Oligotrophen, Heterotrophen, Ammonifikaten, Nitraten und Nitriten, Denitrifikaten und dem Nachweis von Mikromyceten. Um ein komplettes Bild des mikrobiologischen Prozesses dieses Lebensraumes zu erstellen, wurden die bakteriologischen Analysen durch quantitative und qualitative enzymologische Analysen ergänzt. Bei allen sechs Materialproben der Höhle traten folgende quantitative und qualitative Aktivitäten auf: die aktuelle und potentielle Dehydrogenaseaktivität, Katalase und Phosphatase aktivität. Nur bezüglich der Intensität des Prozesses wurden Unterschiede sichtbar. Die qualitativen enzymologischen Analysen: die Amylase, die Dextranasen, die Invertase, die Maltase und Levanase wurden bei den Proben nicht nachgewiesen.
Schlüsselworte: mikrobiologische Analyse, enzymologische Analyse, der Dârninii Höhle.

Rezumat. Au fost analizate microbiologic şi enzimologic șase probe de material cavernicol provenite din Peştera Dârninii, Munţii Bihor. Analizele microbiologice au constat în determinarea prezenţei bacteriilor oligotrofe, heterotrofe aerobe, amonificatoare, nitrat- şi nitrit bacterii, denitrificatoare şi a prezenţei micromicetelor. Pentru a ne forma o imagine completă asupra proceselor microbiene din acest habitat, analizele bacteriologice au fost completate cu analize enzimologice cantitative şi calitative. La toate cele 6 probe de material cavernicol au fost determinate activităţile cantitative: dehidrogenazică actuală şi potenţială, catalazică şi fosfatazică. Au fost înregistrate diferenţe legate doar de intensitatea procesului enzimatic. Activităţile enzimatice calitative: amilazică, dextranazică, zaharazică, maltazică şi levanazică nu au fost prezente la probele studiate.
Cuvinte cheie: analize microbiologice, analize enzimologice, Peştera Dârninii.

Introduction. Dârninii Cave is situated in Bihor Mountains, at the origin of Albac Valley, in the right slope, at an altitude of about 1220 meters. The cave has a 5645 meters development, with an 810 meters extension and 112 meters height. The aria in which the cave developes belongs tectono-structurally to the Bihor autochton. The morphology of the cave shows the mechano-tectonic conditioning of the local karst and the whole cavity is developed in limestone and landinian anisian triassic dolomite of Bihor autochton, on two levels: one fossil and one active (Bleahu et al 1976).

The biggest stalagmitic dome in Romania, over 10 meters high, a real "mammoth" is found in this cave. His reds and browns are mainly due to iron hydroxides and organic matter. The whites are pure calcite (Onac & Kearns 2000).

The richness in speleothems on the fosile level suggests the Dârninii Cave is an ancient cave (see Figure 1).

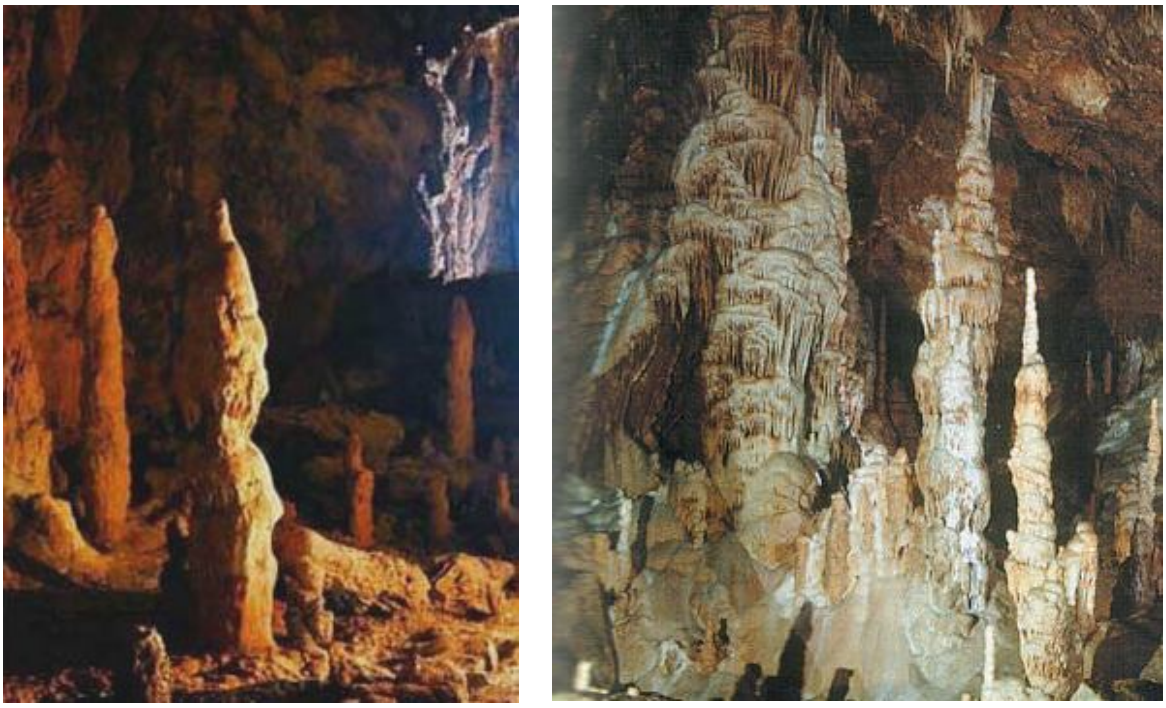

Figure 1. Images from Dârninii Cave.

Samples were collected from this cave and subjected to microbiological and enzymological analyses. The sampling sites are described in Figure 2 and the cave material samples for microbiological and enzymological analyses were collected as follows:

Sample 1 – floor clay, close to the metal gate; many *Pholeuon sp.* individuals were found in this zone.

Sample 2 – floor clay, Bivuac Hall, at the very end of the hall.

Sample 3 – wall clay, at the entrance of Impressionists's Gallery, crossway with Mammoths's Gallery; the recorded sediment temperature on this spot was 5.7 ºC.

Sample 4 – moonmilk from the wall, at the entrance of Impressionists's Gallery, very close to the sampling spot of Sample 3; the recorded temperature on this spot was also 5.7 ºC.

Sample 5 – clay from a more sheltered place situated between floor and wall; this zone harbored many bat bones and *Pholeuon sp.* The temperature recorded in this site attained 6 ºC.

Sample 6 – moonmilk from a formation, from Impressionists's Gallery, place where more *Pholeuon sp.* individuals were identified.

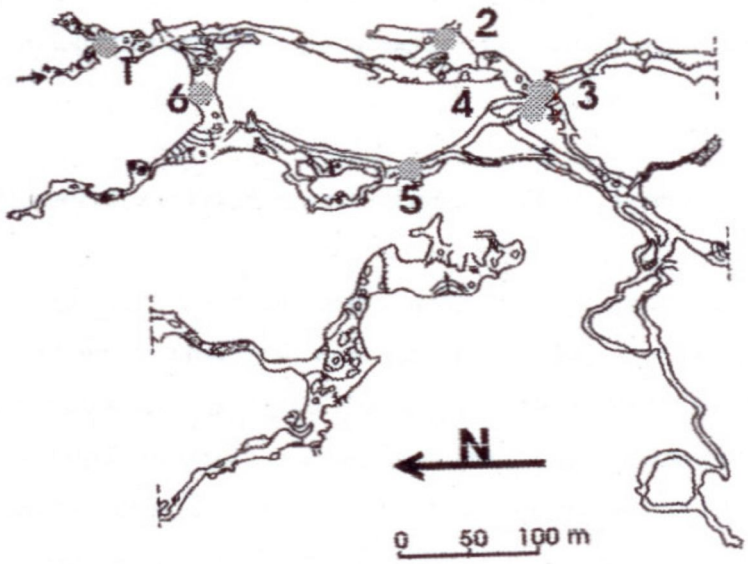

Figure 2. The map of Dârninii Cave and the sampling sites.

Material and Methods

Microbiological Analyses. There was determined the number of bacteria belonging to the following ecophysiological groups: oligotrophic bacteria, aerobic mesophilic heterotrophs, ammonifiers, denitrifiers, nitratebacteria and nitritebacteria. All operations connected to the bacteriological determinations were carried out under sterile conditions.

The *oligotrophic bacteria* were determined on the medium described by Hattori & Hattori (1980). The presence of the *aerobic mesophylic heterotrophs* was determined with bullion agarized medium on plates (Atlas 2004). After incubation the number of colonies in each Petri dish was counted, the average of the parallel samples values was calculated from the most significant dilution and it was multiplied with the reverse value of the respective dilution. The analysis of the *ammonifiers* was performed on peptone medium. For *denitrifiers* the De Barjac culture medium (Pochon 1954), and for *nitrate- and nitritebacteria* a selective liquid medium (Allen 1957) was used.

The culture media were inoculated, by inoculation loop, directly with cave material.

Incubation took place at 28 ºC in all the cases. The nitrate-, nitritebacteria and denitrifiers cultures were incubated for 14 days and the other cultures for 7 days.

The cultures were examinated macroscopically, microscopically and/or chemically. Macroscopically was considered the presence of the colonies, their shape, colour, margin, surface and aspect, the consistence, transparence or opacity of the colonies. Microscopical examination implied making smears on slides, utilised in the Gram coloration method. Regarding Gram coloration the bacteria split in Gram positives, Gram negatives and Gram variables. Gram positive bacteria turn violet, Gram negative bacteria turn red and the variable ones can turn either way (Drăgan-Bularda 2000).

In order to determine the presence of mycromicetes Czapek-Dox medium was used (Drăgan-Bularda 2000). This medium was examinated only macroscopically, noting only the presence or absence of mycromicetes cultures.

The other cultures were chemically examinated, assaying the presence of ions or compounds resulting from the bacterial metabolism. Ammonifiers were analysed using Nessler reagent in order to assay the ammonia produced. The presence of yellow-orange coloration on the inoculated medium and its absence on the non-inoculated one shows ammonification, meaning the presence of these bacteria in culture medium. The nitritebacteria cultures were analysed using diphenylamine reagent. The turning blue

means nitrites and nitrates are present in the cultured medium. The cultures of *denitrifiers* were examinated with Griess I and Griess II reagents. The pink or red coloration shows the nitrite presence and proves that nitrates were reduced to nitrites.

***Enzymological Analyses*.** The enzymological analyses were both quantitative and qualitative. *The quantitative analyses* consisted in determination of the following enzymatic activities: catalase (Kappen 1913), phosphatase (Krámer & Erdei 1959), actual and potential dehydrogenase (Casida et al 1964).

Catalase activity was determinated by Kappen method and is expressed in H_2O_2 mg. Phosphatase activity was determined in reaction mixtures preparated from 5 g of cave material and 10 ml 0.5% disodium phenylphosphate solution. It is expressed in mg phenol/5 g cave material (Drăgan-Bularda 2000). Dehydrogenase activity was assessed using Casida et al method (1964). In order to determine the actual dehydrogenase activity the reaction mixtures contained 3 g cave material + 0.5 ml 3% TTC (2,3,5-triphenyltetrazolium chloride) solution + 2 ml distilled H_2O. For determining the potential dehydrogenase activity instead of 2 ml distilled H_2O only one ml was added + 1 ml 3% glucose solution. Incubation took place at 37 ºC, during 72 hours. The dehydrogenase activity is expressed by mg of triphenyl-formasane.

At every determination of enzymatic activity control reaction mixtures without substrate (without cave material) were prepared.

The qualitative analyses consisted in determination of the following enzymatic activities: saccharase, amylase, maltase, dextranase and levanase. The technique used to assay these enzymatic activities was paper circular chromatography. The reaction mixtures consisted of 3 g cave material + 2 ml toluene (for preventing the proliferation of microorganisms) + 5 ml 2 % enzymatic substrate (saccharose, starch, maltose, dextrane and levane); incubation 7-14 days at 37 ºC. After developing the chromatographic paper, the reductive hydrolytic products were emphasized. The larger spots for the hydrolytic products show the higher enzymatic activities (Drăgan-Bularda 2000).

Results and Discussion

Results of Microbiological Analyses. The ammonifiers are present in all analysed samples, with difference only in the reaction intensity. These different intensities were noted by +, more + meaning higher intensity (Table 1). Table 1 shows that nitratebacteria were present quite abundantly in most of the samples, the smallest number being recorded in sample 3. The nitritebacteria presented a small density, with absence in sample 6. The least present were denitrifiers, missing in all the samples except sample 1.

Table 1

Presence/absence of nitrogen cycle bacteria

	Sample	*Ammonifiers*	*Nitratebacteria*	*Nitritebacteria*	*Denitrifiers*
Dârninii Cave	1.	+ +	+ + +	+	+
	2.	+	+ ++	-	-
	3.	+ +	+ +	+	-
	4.	+ + +	+ + +	+	-
	5.	+ + +	+ + +	++	-
	6.	+ +	+ + +	+	-

These bacteria prove the existence of nitrogen cycle in the subterranean ecosystem. In order to complete the food chain and for explaining the nitrogen circulating mode two more links, namely the nitogen fixing bacteria and the nitrit- and nitrat- decomposing bacteria, are needed, conclusively the first and the last chain link (Figure 3).

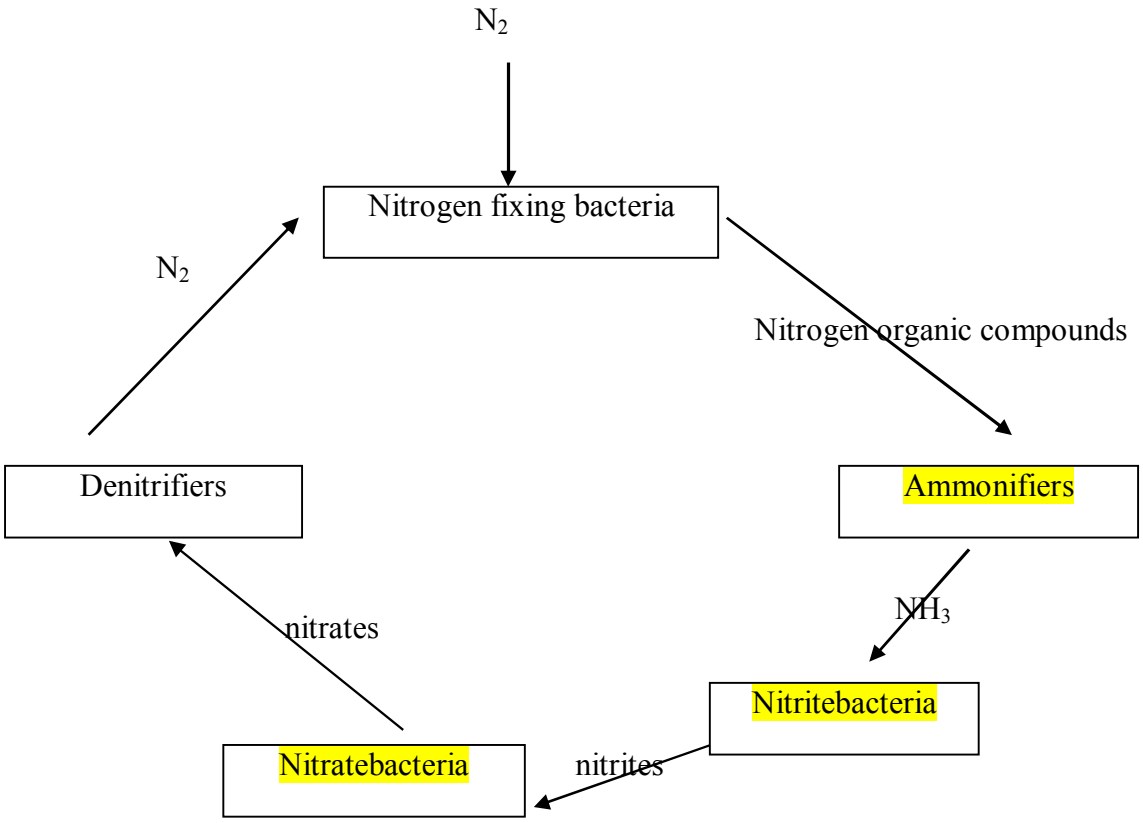

Figure 3. Food chain prefigurating nitrogen cycle in subterranean ecosystems. The colored boxes indicated the links evidenced in our samples.

At the samples collected from Dârninii Cave the medium for oligotrophs was prepared in 3 forms: undiluted, 10x dilution and 100x dilution. It was noticed that most colonies formed on undiluted medium, their number diminishing proportionally with dilution increasing. So that on the 100x diluted medium there were no colonies. The colonies developed as follows: sample 1 – the colonies appeared both on undiluted and 10x diluted medium; sample 2 and 4 – only on undiluted medium; the rest of the samples – no colonies (Tabel 2). The medium for heterotrophs developed no colonies.

The colonies were macroscopically examinated:

Sample 1. On undiluted medium 3 types of colonies were obtain: (a) small yelow points, 0.2-0.3 cm in diameter, well defined margins, smooth and slightly convex surface; (b) big white spots, 1.5-2 cm in diameter, undulated margines, spongy look; (c) brown-whitish small spots, 0.2-0.5 cm in diameter, strait margins, slightly convex surface. On 10x diluted medium a single colony was present with a more intense white spot in the centre and thinning towards the edges.

Sample 2. A single whitish colony covering all the medium surface.

Sample 4. A single colony looking randomly extended and covering about half of the medium surface, with clear margins and smooth surface.

By microscopical examination of the smears from the cultures it was observed that most microorganisms have a bacillary form (Figure 4). The dimensions of these rods are between 1 and 10 µm. Generally, the rods are separated but streptobacilli were also observed. Bacterial spors were also present meaning that some of these bacteria are sporulating. Cocci, Gram positive, very small, were also observed and they formed yellow colony. The yeast are also present on some smears.

Table 2

The presence of microorganisms on the medium for oligotrophs in Dârninii Cave

Samples	*Medium dilution*		*Microbial groups*	*Gram coloration*	*Dimension (μ)*
1.	Undiluted	(a)	cocci	+	1
			yeasts	+	-
		(b)	bacilli	+	2.3-3.5
			bacterial spors		1
			streptobacilli	+	1.5-3
			yeasts	+	-
		(c)	bacilli	-	1-2
	1/10 diluted		bacilli	+	≤1
			bacterial spors		1.5
			yeasts	+	-
	1/100 diluted		-		
2.	undiluted		bacilli	+	2
			yeasts	+	-
	1/10 and 1/100 diluted		-		
4.	undiluted		streptobacilli	+	2.5-5
			bacilli	+	4-7
			filamentous bacilli	-	-
			bacterial spors		-
	1/10 and 1/100 diluted		-		

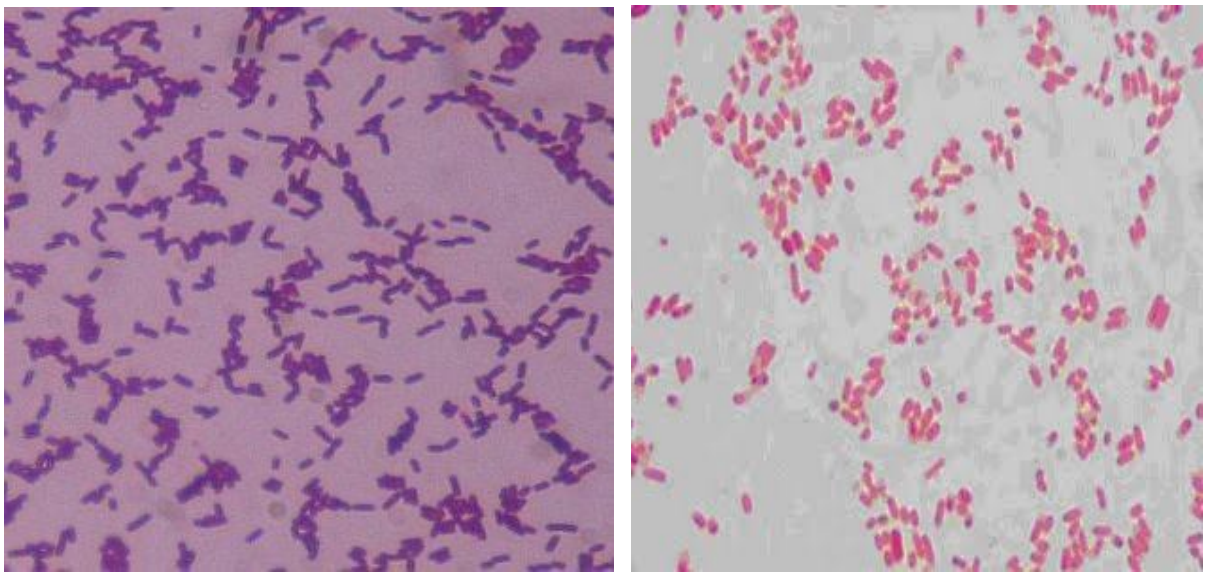

Figure 4. Oligotrophs from Dârninii Cave under microscope (100x).

The microscopical analyses were performed in order to detect the presence of the bacterial groups presented above. It came out that they are present even though in some cases in small number.

Although no specific cultured medium for yeast was used they were recorded, on undiluted oligotroph medium.

Results of Enzymological Analyses. Enzymological analyses proved the presence of life in this cave at the moment of the analyses and a long time before it. The dehydrogenase activity is due to the enzymes contained by the proliferating

microorganisms as catalase and phosphatase activities are due to the accumulated enzymes (Zborovschi et al 1989). The presence of dehydrogenase activity denotes the presence of living microorganism at the sampling moment and the other two activities indicate their persistence in time. Catalases and phosphatases come from the lysate cells, they are absorbed on the organo-mineral colloids and accumulate (Paşca et al 1993). These enzymes point out the biological potential of the cave material.

The dehydrogenase activity had lower values but it was present (Table 3). The actual dehydrogenase activity had the lowest value due to the small amount of organic substances in the cave environment. The potential dehydrogenase activity was higher because a nutritional source (glucose) was added.

Table 3

Enzymatic activities in Dârninii Cave

Sample	*Dehydrogenase activity (mg. triphenyl - formasane)*		*Catalase activity (mg H_2O_2)*	*Phosphatase activity (mg phenol)*
	Actual	Potential		
1.	0.007	0.015	0.2	0.043
2.	0.043	0.083	0.3	0.053
3.	0.102	0.106	0.5	0.084
4.	0.105	0.112	0.4	0.079
5.	0.012	0.032	0.6	0.056
6.	0	0	0.3	0.009

Figure 5 shows that at 4 out of 6 samples the actual dehydrogenase activity is smaller than the potential one. The figure also displays how these activities vary according to the cave depth where the samples were collected, without a corelation between the values and the depth.

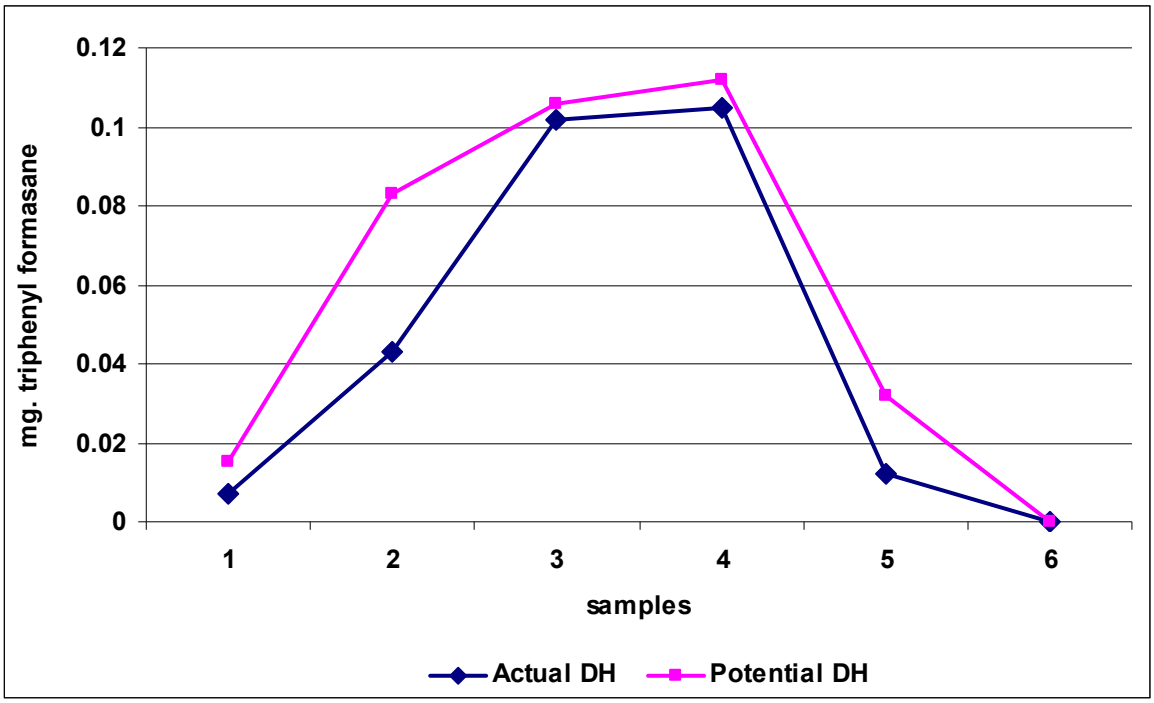

Figure 5. Actual and potential dehydrogenase activity in Dârninii Cave.

For catalase and phosphatase activities graphics were made in order to observe how they vary according to the cave depth they were collected from (Figs 6-7).

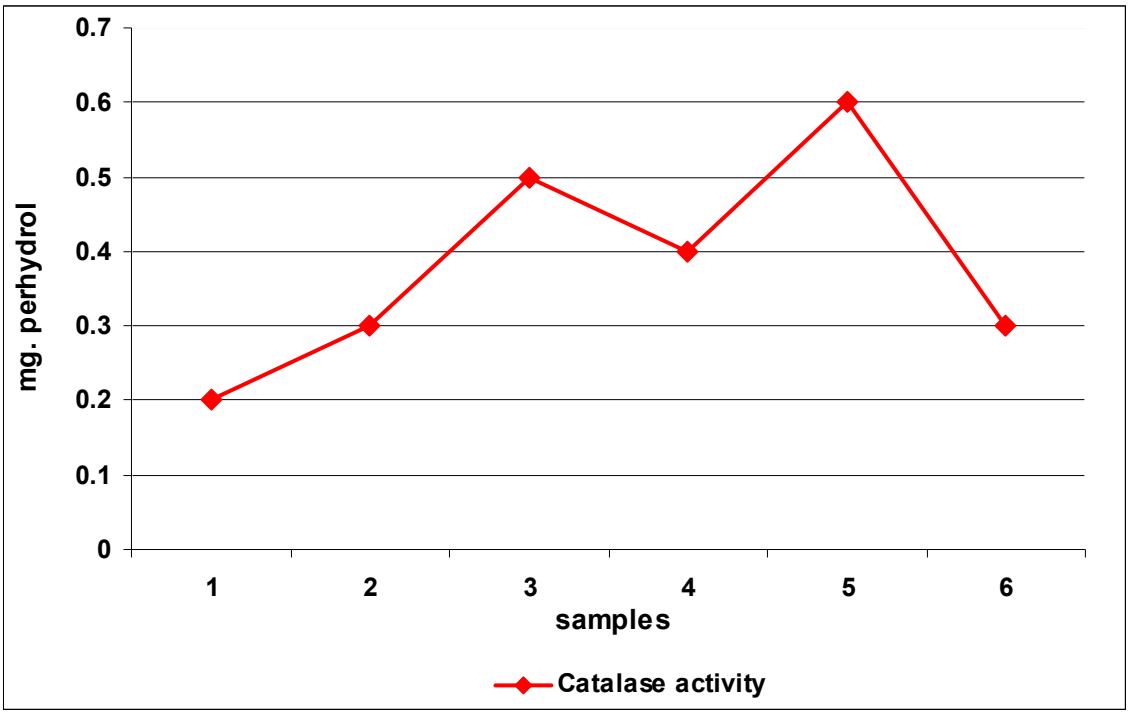

Figure 6. Catalase activity in Dârninii Cave.

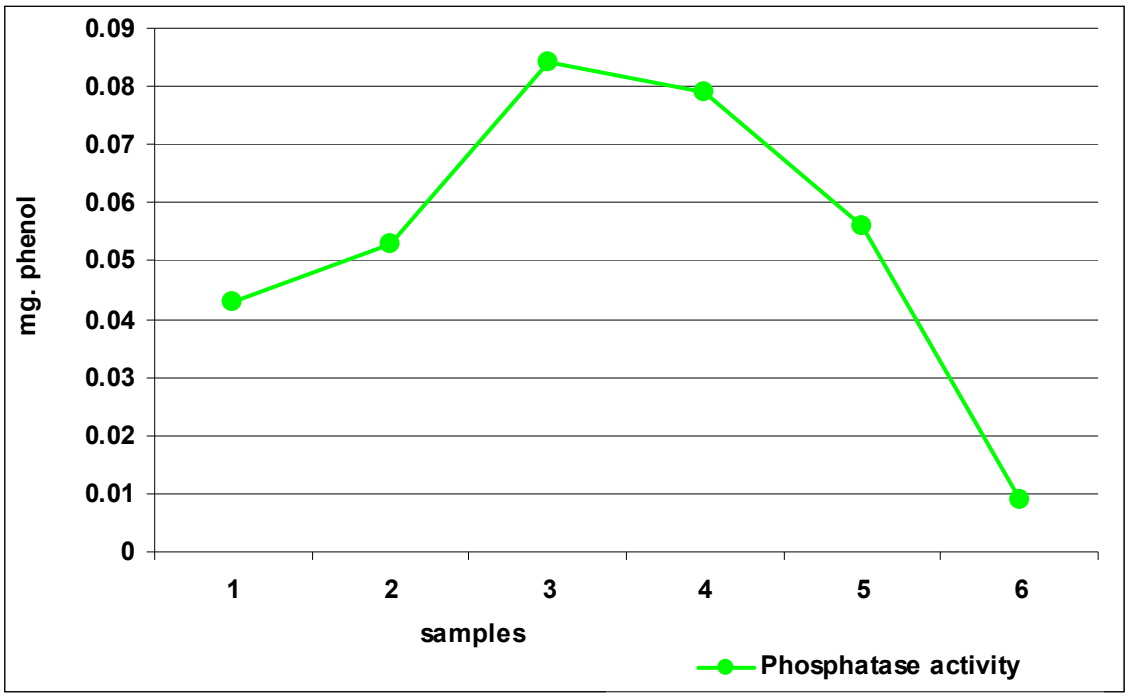

Figure 7. Phosphatase activity in Dârninii Cave.

As we move away from the entrance of the cave these activities should diminish because the organic substances contribution from outside lowers. But these graphics shows a different picture. Kiss et al (1991), in a study on cultivated soils, concluded that enzymatic activities are influenced by more factors including: soil loosening, aeration grade and also the nature of the present organic and mineral substances. It seems that

the same multifactorial situation applies here, the organic substance quantity being unable to explain the variation.

Qualitative enzymatic analyses were also performed (saccharase, amylase, maltase, dextranase and levanase) but the results were negative.

Conclusions

The presence of microorganisms in the Dârninii Cave environment was pointed out by microbiological and enzymological analyses.

The next bacterial groups, capable of multiplying on the culture media, were microbiologically identified: oligotrophic bacteria, aerobic mesophilic heterotrophs, ammonifiers, denitrifiers, nitrifiers (nitratebacteria and nitritebacteria).

On specific oligotrophs medium microbial colonies were obtained from most of the analysed samples. This is according to the cave being an oligotroph environment.

For the same samples tested for oligotrophs the aerobic heterotrophs were also tested but with negative outcome. This indicates that the oligotrophs from the cave are strictly oligotrophs, adapted to the oligotroph cave environment and native to the cave.

The presence of the ammonifiers, nitrate and nitritebacteria in some samples may suggest the existence of a food chain. The same bacteria indicate the nitrogen cycle in the subterranean environment.

In the samples from Dârninii Cave yeast were found, only by microscopically analyses, although no specific culture medium was used.

The micromycetes were not present in the analysed samples.

The enzymological analyses pointed out the presence of enzymes from live microorganisms and also enzymes accumulated on organo-mineral colloids. These analyses reflect the biological potential of the environment and no differences were found dependent on the distance from the entrance.

The dehydrogenase activity indicates the presence of enzymes from proliferating microorganisms. The actual dehydrogenase activity is low because the organic material quantity in the cave environment is very small. The potential dehydrogenase activity is bigger due to the glucose added.

The catalase and phosphatase activities are developed by the enzymes absorbed on organo-mineral colloids. These proves the presence of microorganisms on a long time period.

The qualitative enzymatic analyses gave negative results.

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