COMPLEX RESEARCHES IN SOUTHERN DOBROGEA, WITH SPECIAL REFERENCES TO THE MOVILE REGION

(SECOND PART)

NEW CONTRIBUTIONS TO THE MICROBIOTA NATURALLY OCCURRING IN THE MOVILE CAVE

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In September 2000, nearly 15 years after the Movile cave (Mangalia, Romania) was discovered and it was demonstrated that this ecosystem is entirely chemoautotrophically based and thus able to sustain the energy requirements of all its components, a new campaign for sample collection was carried out. The sample collected were microbiologically and biochemically analysed using selective media for a large range of physiological groups of chemoautotrophic and heterotrophic microorganisms. It was possible to get new data concerning the naturally occurring microorganisms.

Key words: subsurface microbiology, chemoautotrophic bacteria, sulfur-oxidizing bacteria, Movile cave ecosystem.

1. INTRODUCTION

The Movile cave was discovered in June 1986, when an artificial shaft dug at the edge of "Obanul de la Movile" sinkhole intercepted a natural cave passage that never had a natural entrance (CONSTANTINESCU, 1989). Two levels were explored: a 200 m long dry level and a 40 m long submerged level (CONSTANTINESCU, 1989; LASCU, 1989; BOGHIEAN and RACOVIŢĂ, 1989). In order to protect the inside of the cave from the possible invasion of surface species as well as to preserve the physico-chemical characteristics of the cave atmosphere, gates were installed at the surface and at the bottom of the shaft. The deepest gate is airtight and it should allow the cave to reestablish the original atmospheric conditions present before it was intercepted by the artificial shaft.

The gaschromatographic analysis of the chemical composition of the cave atmosphere indicated that the amount of carbon dioxide increases in the vicinity of the lake and that the remote air bells contain very high amounts of CO_2. Oxygen concentration reach values as low as 10% in the remote air bells, where small concentrations of methane were also found. The elevated CO_2 levels in the deep hypogean environment associated with the thermomineral sulfurous waters may have influenced the evolution of the troglomorphic fauna (SÂRBU and POPA, 1992). These

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authors consider that the entire energy base of the biological community appears to be supplied by autochthonous chemolithoautotrophic carbon fixation by sulfur-oxidizing microorganisms. Thus, the trophic source for the communities living in such habitat as the Movile cave is the chemoautotrophic production of the naturally occurring microbiota. The above mentioned authors underline that a thick microbial mats cover the water surface and lime-stone walls of the cave, and the preliminary studies indicate that these mats are composed of chemoautotrophic organisms which use hydrogen sulfide rich in the cave, as an energy source. It is considered by SÂRBU and POPA (1992) that this chemoautotrophically based cave ecosystem appears that relies exclusively on autochthonous primary production. The microbes living in the waters of the Movile cave area, use hydrogen sulfide as an energy source and oxygen as the final electron acceptor. Besides elemental sulfur, they also produce sulfuric acid as a metabolic bioproduct. Consequently, the subterranean waters become more aggressive when they reach water-table and become colonized by sulfur-oxidizing bacteria. This process could represent the major driving force of the speleogenetic progress in this region.

Microbiological analyses carried out by Dumitru (quitted by SÂRBU and POPA, 1992) and by VLĂSCEANU and DANCĂU (1992) report the occurrence of bacteria such as *Thiobacillus thioparus*, *Thiobacillus ferrooxidans*, *Desulfovibrio* sp., *Beggiatoa* sp. and other bacteria involved in the sulfur cycle or chemolitotrophic bacteria involved in carbon fixation, as well as some fungi belonging to the genera *Gliocladium, Penicillium, Trichoderma* and *Plasmopora.*

It was concluded that the chemolithoautotrophic bacteria living in the water and in the floating mats oxidize hydrogen sulfide and use the resulting energy for carbon fixation. The existence of this process was initially suggested by the discovery of deposits of elemental sulfur within the floating mats. This sulfur results as a bioproduct of the biological sulfide-oxidation that take place in the chemoautotrophic microbiota. With this purpose experimental studies were performed to determine whether the microbiota is indeed chemolithoautotrophic (SÂRBU, 1991; SÂRBU *et al.*, 1991). Such experiments show that the microbiota living in the water column of the Movile cave is also involved in the sulfide-driven chemolitoautotrophic carbon fixation, as well as that the autotrophic bacteria produce and excrete large amounts of organic molecules, that serve as food for the rich populations of heterotrophic fungi and bacteria. It is supposed that this unique subterranean ecosystem is indeed entirely chemolithoautotrophically based and thus able to sustain the energy requirements of all its components.

In the samples collected during Oct. 1993–May 1994 and microbiologically analysed by CHIURTU and DUMITRU, these authors reported in 1995 the occurrence in the Movile environments of several groups of bacteria such as aerobic heterotrophic bacteria belonging to genera: *Sarcina, Serratia, Corynebacterium, Bacillus*; sulfur-oxidizing bacteria belonging to genera *Thiobacillus* (*T. thiooxidans,*

T. thioparus, T. denitrificans) and *Beggiatoa*; sulfate-reducing bacteria belonging to genus *Desulfovibrio*; nitrifying and denitrifying bacteria as well as some iron bacteria.

SÂRBU *et al.* (1994a, 1996), SÂRBU and KANE (1995) and VLĂSCEANU *et al.* (1997) report additional results as a support for the comments mentioned above. Several different autotrophic sulfur-oxidizing bacteria were isolated from the cave water and floating mat. Most isolates were tentatively identified as members of the genera *Thiobacillus* and *Thiosphaera*, and some spiral-shaped and filamentous bacteria were also isolated but not identified. Finally the authors concluded that the Movile cave community is biologically isolated and receives little, if any, organic carbon inputs from the surface environment, as well as that the presence of autotrophic bacteria lends support to the hypothesis that there is a chemoautotrophic base to the Movile cave ecosystem. It was clearly proved that the environment of the Movile cave contain chemoautotrophic bacteria that fix inorganic carbon, using hydrogen sulfide as an energy source. Thus, the analysis of stable carbon and nitrogen isotopes showed that this chemoautotrophic production is the food base for 48 species of cave-adapted terrestrial and aquatic invertebrates, 33 of which are endemic to this cave ecosystem, known to be supported by *in situ* autotrophic production.

Recently, PORTER *et al.* (2000) have underlined that only two caves (the Movile cave, Romania and the Frasassi caves, Italy) have been found to contain higher trophic levels that are entirely dependent on chemoautotrophic microorganisms. The model established by these authors indicates that in the case of the Movile cave:

1) chemoautotrophy is an ecologically significant source of primary production, and
2) the energy flow within the sulphidic karst systems supported by chemoautotrophy has the potential to be utilized extremely efficiently.

Also very recent, MANOLACHE *et al.* (2000), have isolated from samples of cave mat and cave water and characterized the methanotrophic bacteria from the Movile cave considered as an unusual environment.

During 2000, a group of researchers from the Center of Microbiology, Institute of Biology of the Romanian Academy, collected samples from the Movile cave (cave mats and cave waters) for microbiological analyses, for a better understanding of the microbiology of such an unusual environment. As about 15 years passed since the Movile cave was discovered, we paid special attention not only to chemolithoautotrophic physiological groups of bacteria, but also to the large physiological groups of heterotrophic bacteria. The results of these microbiological analyses are presented in this paper.

2. MATERIAL AND METHODS

Microbiological analysis of the samples. In Fig. 1 all sampling points from the Movile cave area, where samples were collected for microbiological and

biochemical analysis are presented. The first series of samples were collected on 7 September 2000 and they were as follows:

- sample 1: surface sulfurous stream, at 300 m distance;
- sample 2: floating surface mat – Bell 2;
- sample 3: surface stream water cave – 5 cm depth;
- sample 4: water cave Bell 2–20 cm depth;
- sample 5: floating surface mat – Bell 1.

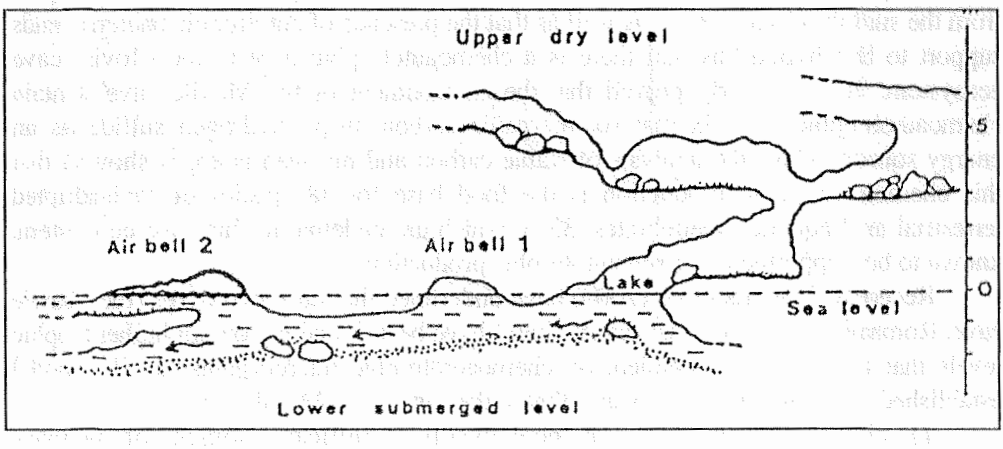

Fig. 1. – Cross-sectional map of the Movile Cave system (after SÂRBU *et al.*, 1994a).

Sample collection for microbiological analysis followed all the adequate requirements. In this series of samples, a number of 20 physiological groups of microorganisms on selective adequate media were studied. In Table 1, the selective culture media used for each physiological group of microorganisms are presented.

The quantitative estimation of microorganisms, naturally occurring in the samples collected on 7 September 2000, was carried out on the basis of the MPN (Most Probably Number) method, according to the McCready statistic tables (RODINA, 1972).

Measurement of the dehydrogenase activity. The method of CASIDA *et al.* (1984) was used with some adaptations for liquid samples. The method is based on the reduction of triphenyltetrazolium chloride (TTC) under the action of dehydrogenase, with formation of formasan, a red compound that can be determined spectrophotometrically (at 485 nm). Dehydrogenase activity is expressed in: mg formasan/ml sample/ 27°C/ 24h.

Table 1

The selective culture media used for each physiological group of microorganisms

No.	Physiological group of microorganisms	Culture media used for isolation	Authors
1	Heterotrophic aerobic and facultative anaerobic bacteria	Nutrient broth Nutrient agar	Williams and Cross, 1971
2	Heterotrophic anaerobic bacteria	VL medium	Beerens, 1953
3	Heterotrophic acidophilic bacteria	Golovacheva medium	Golovacheva and Karavaiko, 1978
		Manning medium	Manning, 1975
4	Metilotrophic bacteria	Bushnel-Hass with methanol medium	Willkinson, 1972
5	Cellulosolytic bacteria	Stapp medium	Rodina, 1972
6	Amilolytic bacteria	Medium with starch	Hulea, 1969
7	Fixation nitrogen bacteria	Ashby medium	Rodina, 1972; Romanenko and Kuznetsov, 1974
8	Nitrite bacteria	Medium for nitrite bacteria	Rodina, 1972
9	Nitrate bacteria	Medium for nitrate bacteria	Rodina, 1972
10	Denitrifying bacteria	Giltay medium	Rodina, 1972
11	Sulfate reducing bacteria	Postgate B medium	Postgate, 1984
12	Sulph-oxidizing chemolithotrophic bacteria (on S^0)	Waksman with S^0 medium, pH 4.0 and 6,6	Waksman and Joffe, 1972
13	Sulph-oxidizing chemolithotrophic bacteria (on $S_2O_3^{2-}$)	Postgate with $S_2O_3^{2-}$ medium, pH 5,0 and 7,5	Postgate, 1966
14	Iron-oxidizing chemolithotrophic bacteria (on Fe^{2+})	9K medium	Silverman and Lundgren, 1959
15	Iron bacteria	Vinogradsky medium	Rodina, 1972
16	Cyanobacteria	BG 11 medium	El-Enany and Issa, 1999
17	Actinomycetes	Gause medium	Williams and Cross, 1971
18	Yeasts	Sabouraud medium	Hulea, 1969
19	Filamentous microscopic fungi	Czapeck- Dox medium	Booth, 1971
		Potato-dextrose-agar medium	Hulea, 1969
20	Unicellular green algae	Knop-Pringhain medium	Sălăgeanu and Atanasiu, 1981

3. RESULTS AND DISCUSSIONS

The results obtained after the **microbiological analysis** of the series of the samples collected on 7 September 2000 expressed in number of cells/ml sample are presented in Table 2.

Table 2

Quantitative estimation of the microorganisms naturally occurring in the Movile cave samples collected on 7 Sept. 2000

Physiological groups of microorganisms	Culture media used for isolation	No. of cells/ml sample				
		Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Heterotrophic aerobic and facultative anaerobic bacteria	Nutrient broth, nutrient agar	7.0×10^7	8.5×10^6	2.5×10^7	4.5×10^7	6.5×10^6
Heterotrophic anaerobic bacteria	VL medium	2.5×10^3	1.5×10^5	7.5×10^5	1.0×10^2	4.5×10^4
Heterotrophic acidophilic bacteria	Golovacheva medium	1.0×10	7.5×10^3	4.5×10	2.5×10	1.5×10
	Manning medium	2.5×10	2.5×10^4	0	0	2.5×10
Metilotrophic bacteria	Bushnel-Hass with methanol medium	2.5×10^4	2.5×10^4	2.5×10^2	2.5×10	2.5×10^4
Cellulosolytic bacteria	Stapp medium	1.0×10	1.0×10^2	0	0	2.0×10
Amilolytic bacteria	Medium with starch	1.5×10^3	3.5×10^5	4.5×10^4	7.5×10^2	3.0×10^4
Fix ation nitrogen bacteria	Ashby medium	3.0×10^4	2.5×10^6	3.5×10^5	2.5×10^4	2.5×10^5
Nitrite bacteria	Medium for nitrite bacteria	7.0×10	0	0	$2,0 \times 10^3$	$2,5 \times 10$
Nitrate bacteria	Medium for nitrate bacteria	9.0×10^2	9.5×10^4	1.2×10^3	4.5×10^4	2.0×10^4
Denitrifying bacteria	Giltay medium	1.5×10^3	9.5×10^2	4.5×10^2	1.5×10	1.5×10^3
Sulfate reducing bacteria	Postgate B medium	4.5×10^5	2.0×10^4	9.5×10	9.5×10	1.5×10^3
Sulph-ox idizing chemolithotrophic bacteria (on S^0)	Waksman with sulphurous (pH=4.0)	2.5×10	2.5×10^4	4.5×10	4.5×10	4.5×10^2
	Waksman with sulphurous (pH=6.6)	9.5×10^5	2.5×10^5	2.0×10^3	2.5×10	4.5×10^4
Sulph-ox idizing chemolithotrophic bacteria (on $S_2O_3^{2-}$)	Postgate with $S_2O_3^{2-}$ (pH=5.0)	4.5×10^3	2.5×10^4	2.5×10^2	1.5×10^2	1.5×10^4
	Postgate with $S_2O_3^{2-}$ (pH=7.5)	4.5×10^6	2.0×10^5	1.5×10^3	9.5×10	2.5×10^5
Iron-ox idizing chemolithotrophic bacteria (on Fe^{2+})	9 K medium	0	4.0×10	0	0	2.5×10
Iron bacteria	Vinogradsky medium	7.5×10^5	1.0×10^4	2.5×10^3	1.6×10^2	6.0×10^4
Cyanobacteria	BG 11 medium	++++	0	0	0	+++
Actinomycetes	Gause medium	1.3×10^3	2.0×10^2	1.0×10^2	2.5×10^2	6.0×10^2
Yeasts	Sabouraud medium	9.0×10	0	1.7×10^3	0	3.7×10^3
Filamentous microscopic fungi	Potato-dextrose-agar medium	7.0×10^3	5.0×10^5	0	2.5×10^2	2.0×10^4
	Czapeck-Dox medium	4.5×10^3	8.5×10^5	1.5×10^2	1.0×10^2	
Unicellular green algae	Knop-Pringhain medium	++++	0	0	0	

The pH values of the 5 water samples from the Movile cave are as follows:

- sample 1 (surface sulfurous stream, at 300 m distance): pH = 7.60
- sample 2 (floating surface mat – Bell 2): pH=6.10
- sample 3 (surface stream water cave – 5 cm deepness): pH = 7.18
- sample 4 (water cave Bell 2–20 cm deepness): pH = 7.25
- sample 5 (floating surface mat – Bell 1): pH=6.78.

The largest diversity of the microbiota naturally occurring in those five analyzed samples is represented by several physiological groups of microorganisms such as bacteria including cyanobacteria and actinomycetes, yeasts, filamentous microscopic fungi and microscopic algae, some of them involved in the natural circulation in nature of carbon, nitrogen, sulfur and iron. The density of the cells on 1 ml sample is as follows:

- Heterotrophic aerobic and facultative anaerobic bacteria: 6.5×10^6–7.0×10^7
- Heterotrophic anaerobic bacteria: 1.0×10^2–7.5×10^5
- Heterotrophic acidophilic bacteria: 1.0×10–2.5×10^4
- Metilotrophic bacteria: 2.5×10–2.5×10^4
- Cellulosolytic bacteria: 0–1.0×10^2
- Amilolytic bacteria: 7.5×10^2–3.5×10^5
- Fixation nitrogen bacteria: 2.5×10^4–3.5×10^5
- Nitrite bacteria: 0–2.0×10^3
- Nitrate bacteria: 9.0×10^2–9.5×10^4
- Denitrifying bacteria: 1.5×10–1.5×10^3
- Sulfate reducing bacteria: 9.5×10–4.5×10^5
- Sulfur-oxidizing chemolithotrophic bacteria: 2.5×10–4.5×10^6
- Iron-oxidizing chemolithotrophic bacteria: 0–4.0×10
- Iron bacteria: 1.6×10^2–7.5×10^5
- Cyanobacteria: 0 – ++++
- Actinomycetes: 1.0×10^2 – 1.3×10^3
- Yeasts: 0–3.7×10^3
- Filamentous microscopic fungi: 1.0×10^2–9.0×10^5
- Unicellular green algae: 0 – ++++

Analyzing comparatively these data, it is easy to detect the physiological groups of microorganisms which are potentially involved in the natural cycles of carbon, nitrogen, sulfur, iron, as well as in the primary production chemolithotrophically based.

The occurrence in some samples of nitrifying bacteria could be due to the seepage from the surface. Unfortunately on the surface area of the Movile cave some solid wastes of the Mangalia town are deposited. The relief of the surface of the Movile cave is under heap forms and at the base of such heaps (knolls) important quantities of water can be accumulated as a source of seepage (Figs. 2 and 2a). In the

matter additional analyses should be done. The phototrophic microorganisms such cyanobacteria and some microscopic algae were detected only in the samples 1 (surface sulfurous stream) and 5 (floating surface mat – Bell 1).

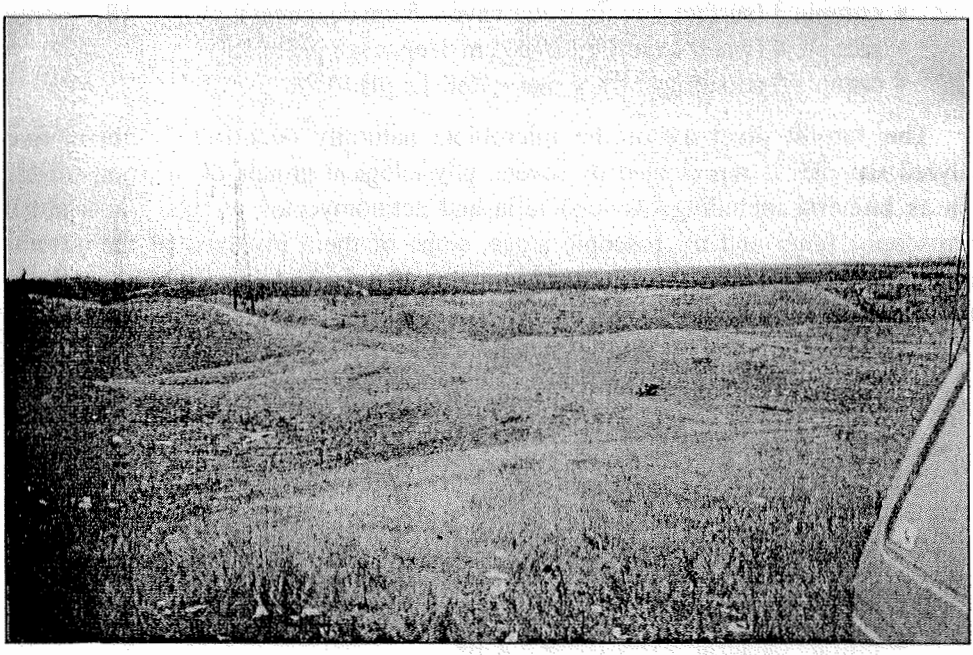

Fig. 2. – The surface area relief of the Movile Cave – Mangalia.

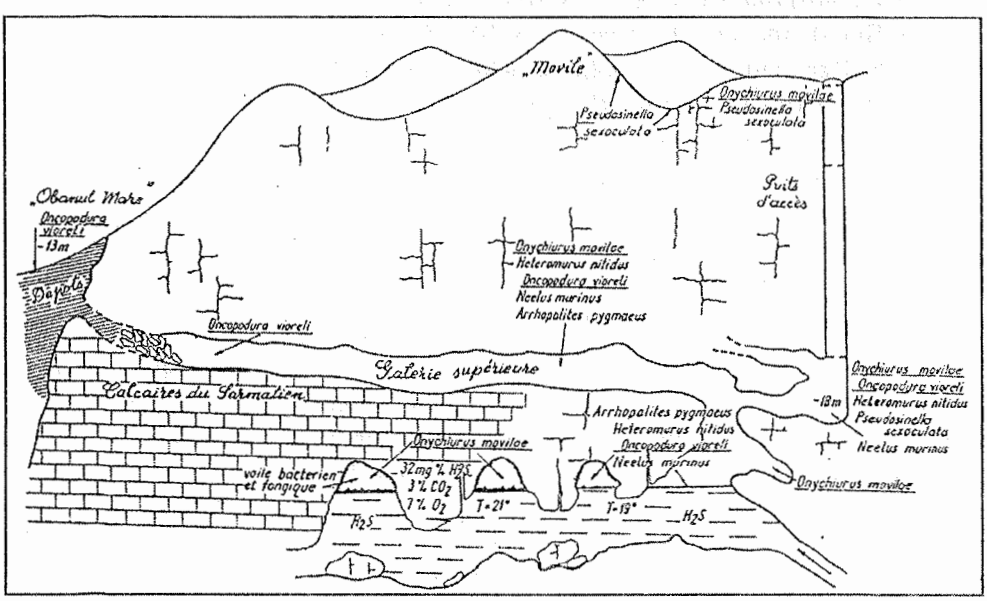

Fig. 2a. – The surface area relief of the Movile Cave – Mangalia (after Gruia 1996).

The H_2S produced at several intensities in all the analyzed samples could be, at least partially of biogenic origin, since the sulfate-reducing bacteria isolated proved high ability to produce H_2S. The intensity (+ ⟶ ++++) of H_2S production (detected using paper bands impregnated in lead acetate), was noticed as follows:

- sample 1 (surface sulfurous stream, at 300 m distance): ++
- sample 2 (floating surface mat – Bell 2): ++++
- sample 3 (surface stream water cave – 5 cm depth): ++++
- sample 4 (water cave Bell 2 – 20 cm depth): +
- sample 5 (floating surface mat – Bell 1): +

The dehydrogenase activity of a sample is relevant for the whole microbial activity, being directly correlated with the living microorganisms in the sample (Table 3).

Table 3

The dehydrogenase activity (mg formazan/ml sample/27°C/24h)

Samples	Dehydrogenase activities:	
	actual	potential
Sample 1 (surface sulfurous stream, at 300 m distance)	8.50	10.20
Sample 2 (floating surface mat – Bell 2)	25.20	27.0
Sample 3 (surface stream water cave – 5 cm depth)	2.20	2.40
Sample 4 (water cave Bell 2 – 20 cm depth)	3.40	5.20
Sample 5 (floating surface mat – Bell 1)	16.80	18.60

Dehydrogenase activities (both actual and potential) of the 5 water samples from the Movile cave showed the presence of rich microbial communities in some cases (sample 2 and sample 5) reaching values similar to those obtained from normal soil samples. In these samples the actual dehydrogenase activity was of 25.2 and 16.8, respectively, mg formasan/ml/27°C/24h, while the potential dehydrogenase activity was of 27.0 and 18.6, respectively, mg formasan/ml/27°C/24h. In the two samples the highest density of microorganisms was detected. The values of the dehydrogenase activities were very well correlated with the results of the microbiological analyses regarding different microbial groups, higher levels of dehydrogenase corresponding to high numbers of microorganisms. In all the cases, the actual dehydrogenase activity was lower than the potential one, due to the lack of specific substrate in each sample, in enough quantity for the high levels of dehydrogenase activities of the samples. On the basis of these results, it can be concluded that the determination of the dehydrogenase activity of a sample is a simple method that can be successfully used to assess the living microorganisms from an environment, at a certain time.

In the previous papers concerning the microbiota of the Movile cave several physiological groups of bacteria were already mentioned as well as some filamentous microscopic fungi. In the samples collected in September 2000, the bacterial groups naturally occurring were much more extended including this time a larger range of microorganisms groups. Also after a number of years the cave was opened, some local conditions changed, and this could be noticed in the high diversity of isolated microorganisms.

4. CONCLUSIONS

1. Nearly 15 years passed since the Movile cave was discovered and it was demonstrated that those 48 species of cave-adapted terrestrial and aquatic invertebrates (33 of which are endemic to this cave ecosystem), are supported by *in situ* autotrophic production by autochthonous chemoautotrophic bacteria which are using hydrogen sulphide as an energy source and oxygen as the final electron acceptor. These autotrophic bacteria produce and excrete large amounts of organic molecules, that serve as food also for the rich populations of heterotrophic bacteria and fungi.
2. The investigations previously carried out concluded that the Movile cave ecosystem is indeed entirely chemoautotrophically based and thus able to sustain the energy requirements of all its components.
3. The results of the microbiological and biochemical analyses of the sample collected on September 7, 2000, are an additional support of the previous investigations mentioned above. Alongside with the active occurrence of several physiological groups of chemoautotrophic bacteria, a large range of several heterotrophic bacteria as well as yeasts and microscopic filamentous fungi are occurring, which finally also serve as food for the rich populations of invertebrates.
4. It was found that the dehydrogenase activity is relevant for the whole microbial activity, being directly correlated with the living microorganisms from the Movile cave environment. No doubt that determination of the dehydrogenase activity is a simple method that can be successfully used to asses the living microorganisms from the Movile cave environment, at a certain time.

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ANNEX 1

Culture media (including ingredients) used for the isolation of several physiological groups, naturally occurring in the Movile cave as well as in the surface sulfuric stream

The medium nutrient broth (g/l):

beef extract	3
peptone	10
NaCl	5
distilled water	1000 ml
pH = 7.2–7.4	

The medium nutrient agar:

the medium has the same composition as nutrient broth, with agar –18 g

The medium VL (g/l):

peptone	1
NaCl	5
beef extract	2
yeast extract	5
glucose	10
chlorhydrate of cysteine	0.5
agar	0.5
distilled water	1000 ml
pH = 7.2–7.4	

This medium has been used both liquid and with agar (18 g/l)

The medium after Golovacheva et al. (g/l):

$(NH_4)SO_4$	0.5
$MgSO_4$	0.3
NaCl	0.2
KH_2PO_4	0.2
$Ca(NO_3)_2$	0.07
yeast extract	0.2
sulfur powder	10.0
distilled water	1000 ml

pH=2.5 adjusted with H_2SO_4;
sterilization at open steams for 1h, for 3 days consecutively

The medium after Manning (g/l):

- Solution 1: sol. of glucose 20% – 25 ml.
- Solution 2:

$(NH_4)SO_4$	1.0
KCl	0.1
$MgSO_4$	0.2
yeast extract	0.5
distilled water	975 ml

pH = 2.8 adjusted with sol. 6 M H_2SO_4

The two solutions were sterilized separately by autoclaving at 121°C for 15 min and then were mixed.

The medium Bushnell-Hass with methanol (g/l):

$MgSO_4$	0.2
$CaCl_2$	0.02
KH_2PO_4	1.0
K_2HPO_4	1.0
NH_4NO_3	1.0
$FeCl_3$	traces
distilled water	975 ml

pH=7.2; sterilization 30 min at 1 atm; after sterilization is added methanol 2.5 ml%

The medium Stapp (g/l):

$NaNO_3$	0.5
$KHPO_4$	0.25
$MgSO_4$	0.1
distilled water	1 000ml

pH = 7.2;

In each tube with medium a band of sterile paper is introduced. Sterilization 30 min at 121°C

The medium with starch (g/l):

K_2HPO_4	0.5
NaCl	0.3
$MgCO_3$	0.3
NH_4Cl	0.5
strach	10.0
distilled water	1000 ml
agar	18.0

pH = 7.2

The medium Ashby (g/l):

K_2HPO_4	0.5
NaCl	0.2

$MgSO_4$	0.2
$FeSO_4$	0.1
$CaCO_3$	5.0
sucrose	18.0
distilled water	1000 ml

pH = 7.6–7.8

The medium for nitrite bacteria:

Vinogradski saline solution (g/l):

K_2HPO_4	0.5
$MgSO_4$	2.5
NaCl	2.5
$Fe(SO_4)_3$	0.05
$MnSO_4$	0.05
distilled water	1000ml

sterilization for 30 min at 121°C

For the preparation of 1 l medium for nitrite bacteria, Vinogradski saline solution, diluted 1:20, 1000ml with (g/l) are well mixed:

$(NH_4)_2SO_4$	0.5
$CaCO_3$	1.0

Sterilization at 110°C for 20 min

To demonstrate the occurrence of nitrite bacteria, a color reaction was used with sulfuric diphenilamine prepared after the recipe:

diphenilamine	0.7 g/l
concentrated H_2SO_4	60 ml
distilled water	1000 ml

To each tube with nitrite bacteria medium and bacterial growth 1 ml conc. H_2SO_4 and 1 ml diphenylamine are added. The occurrence of nitrite bacteria is proved by the appearance of a blue color.

The medium for nitrate bacteria

is represented by a Vinogradski saline solution diluted 1:20, 1000 ml with addition (g/l):

$NaNO_2$	1.0
$CaCO_3$	1.0

Sterilization at 121°C for 20 min.

The occurrence of nitrate bacteria is proved by the addition to each tube with culture media and bacterial growth of a few mg of urea for removal of the nitrites eventually present in medium. Then 1 ml conc. H_2SO_4 and 1 ml diphenilamine reagent are added. The occurrence of nitrate bacteria is demonstrated by the blue color.

The medium Giltay:

- Solution 1(g/l):

asparagine	0.5
glucose	10.0

KNO_3	2.0
distilled water	500 ml

- Solution 2 (g/l):

KH_2PO_4	2.0
Sodium citrate	2.5
$MgSO_4$	2.0
$CaCl_2$	0.2
$FeCl_3$	traces

After separate sterilization, the two solutions are mixed, pH is adjusted to 7.0 using a soluti on 10% of sodium bicarbonate. Then, in each tube, two drops of a solution of bromthymol are added, giving in this way a blue color. The occurrence of denitrifying bacteria is proved by the discolour of the culture medium due to its alkalinity under the action of bacterial metabolisms products.

The medium Postgate P (g/l):

KH_2PO_4	0.5
NH_4Cl	0.1
$CaSO_4$	1.0
$MgSO_4$	2.0
calcium lactate	3.5
tap water	1000 ml

Separately, the following solutions were sterilized:

yeast extract	5%
$FeSO_4$	5%
Na_2S	1%
$NaHCO_3$	10%

The base solution (Postgate medium) is distributed (100 ml) in adequate flasks, and after sterilization, just when it is used for the growth of microorganisms, are added in aseptic conditions to each 100 ml, the following:

yeast extract sol. 5%	2 ml
$FeSO_4$ sol. 5%	1 ml
Na_2S sol. 1%	0.2 ml

$NaHCO_3$ sol. 10% is added till pH becomes 7.2–7.4.

The medium Waksman (g/l):

$(NH_4)SO_4$	0.2
KH_2PO_4	3.0
$MgSO_4$	0.5
$CaCl_2$	0.25
$FeSO_4$	0.01
sulfur powder	10.0
distilled water	1000 ml

Sterilization under open steams for 1h, for 3 days consecutively. This medium was used in two variants, with pH 4.0 and with pH 6.0.

The medium Postgate with $S_2O_3^{2-}$ (g/l):

$Na_2S_2O_3$	5.0
$(NH_4)SO_4$	4.0
KH_2PO_4	4.0
$MgSO_4$	0.5
$CaCl_2$	0.25
$FeSO_4$	0.01
distilled water	1000 ml

Sterilization at 0.5 atm for 20 min. This medium was used in two variants: with pH 5.0 and with pH 7.5

The medium 9K:

– Solution 1 (g/l):

$(NH_4)SO_4$	3.0
KCl	0.1
K_2HPO_4	0.5
$MgSO_4$	0.5
$Ca(NO_3)_2$	0.01
distilled water	700 ml

– Solution 2 (g/l):

$FeSO_4 \times 7H_2O$	44.2
distilled water	300 ml
H_2SO_4 sol. 10 N	1.0 ml

Solutions 1 and 2 are separatly sterilized as follows: sol. 1 at 1 atm. and sol. 2 at 0.5 atm for 20 min. Before use the solutions are mixed.

The medium Vinogradski (g/l):

NH_4NO_3	0.5
$NaNO_3$	0.5
K_2HPO_4	0.5
$MgSO_4$	0.5
$CaCl_2$	0.2
citrate feriamonium	10.0
distilled water	1000 ml
pH = 7.2 – 7.4	

The BG 11 medium (g/l):

$NaNO_3$	15.0
K_2HPO_4	0.4
$MgSO_4$	0.75
$CaCl_2$ cryst.	0.36
acid citric	0.06
citrate feriamonium	0.06
Na EDTA	0.01
Na_2CO_3	0.2

sol. of microelements	1 ml
distilled water	1000 ml

pH = 7.3;
sterilization at 1 atm for 20 min.

Sol. of microelements (g/l):

$B(OH)_3$ or H_3BO_3	2.86
$MgCl_2 \times 4H_2O$	1.81
$ZnSO_4 \times 7H_2O$	0.22
$Na_2MoO_4 \times 2H_2O$	0.39
$CuSO_4 \times 5H_2O$	0.079
$Co(NO_3)_2 \times 6H_2O$	0.049

The medium Gause (g/l):

KNO_3	1.0
K_2HPO_4	0.5
$MgSO_4$	0.5
NaCl	0.5
$FeSO_4$	0.01
starch	20.0
agar	20.0
distilled water	1000 ml

pH = 7.0; sterilization at 121°C for 20 min.

The medium Sabouraud (g/l):

peptone	10.0
glucose	25.0
agar	18.0
distilled water	1000 ml

pH = 5.5–6.0; sterilization at 115°C for 20 min. or at 0.7 atm for 1h.

The medium potato-dextrose-agar (g/l):

potato	250
dextrose	20.0
agar	15.0
distilled water	1000 ml

The slides of potato are boiled in 1 l of distilled water for 1 h, then the remaining liquid is brought to 1000 ml; agar and glucose are added; sterilization at 0.7 atm for 1 h; pH is adjusted to 5.5–6.6. To the media used for the isolation of filamentous microscopic fungi a solution of Streptomycine (30 γ/ml medium) was added for the inhibition of growth of bacteria occurring in the sample.

The medium Czapek-Dox (g/l):

$NaNO_3$	3.0
KCl	0.5
$MgSO_4$	0.01

$FeSO_4$	0.01
$KHPO_4$	1.0
sucrose	30.0
distilled water	1000 ml

pH = 6.5; sterilization at 121°C for 15 min.

The medium Knop Pringhaim (g/l):

KNO_3	1.0
$Ca(NO_3)_2$	0.1
$MgSO_4$	0.1
K_2HPO_4	0.2
$FeCl_3$	0.001
distilled water	1000 ml

pH=7.0; sterilization at 121°C for 20 min.

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