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A comparative study on the microbial activities in some caves from Pădurea Craiului Mountains

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Abstract. Microorganisms represent a heterogeneous group, widely spread in different environments. Our goal in this study was to determine whether microorganisms are present in four particular Transylvanian caves, which are: Ungurului Cave, Izvor Cave, Lesianei Cave, and Moanei Cave, all situated in the Pădurea Craiului Mountains. All of those caves are often visited by tourists. In order to conceive this study we have collected and analyzed different samples, using enzymatic and microbiological methods; the samples were taken from the floor deposits, wall deposits and sludge. Some enzymatic activities were studied, such as: catalase activity, phosphatase activity, actual and potential dehydrogenase activity, urease activity as well as the non-enzymatic catalytic activity. We have also computed the EISQ – which is the enzymatic indicator of soil or sludge quality – based on the results obtained by studying the enzymatic activities.

Keywords: *sludge, wall deposits, enzymological research, Enzymatic Indicator of the Sludge Quality (EISQ), enzymatic potential.*

Introduction

Procaryots can live in an enormous variety of ecological niches, and they are amazingly varied in their biochemistry, far more varied than eucaryotic cells are. There are organotrophic species that can make use of practically any type of organic molecule as food, from sugars or amino acids, to methane. Than, there are the phototrophic species, which can capture light energy in a variety of ways, some of them generating oxygen, others not. Last, but not least, there are lithotrophic species that can feed on a plain diet of inorganic nutrients, getting their carbon from CO_2, and their energy from H_2S, H_2, Fe^{2+}, elemental sulfur or any other chemicals in the environment. A large part of this world of microscopic organisms is virtually unexplored. Traditional methods of bacteriology have allowed us to detect only those species that can be isolated and cultured in the laboratory. But new techniques, such as DNA sequence analysis, have shown that most species can't be cultured by standard laboratory methods. According to one estimate, at least 99 % of procaryotic species are still unknown.

Very few studies have been made as far as the cave microbiota is concerned, mostly because of the complications met when collecting the samples, but also because it is very difficult to establish the origin of the detected bacteria (many of them are allochthonous and accidentally brought in by tourists).

Cave environments are quite steady when regarding the temperature (usually constant), high humidity, as well as the complete lack of light. This particular environment has been very little explored, concerning its potential for new medical products, maybe because some publications during 1950-1960 wrongly stated that caves exhibit a sterile potential in this matter. But, between 1980 and 1997, a large diversity of bacteria was discovered, exploring cave like Lachuguilla (New Mexico), Mammoth (Kentucky, USA), and caves from Hawaii, collecting and culturing over one thousand species.

A large variety of bacteria belonging to the actinobacteria (actinomycetes), cyanobacteria, and even archaebacteria have been isolated from caves. Some actinomycetes give off a characteristic smell of cave entry, due to the production of an original metabolite (geosmine). Cañavaras et all. give a big importance to some actinomycetes species (*Streptomyces roseoviridis, S. flavogriseus etc*) in the process of moonmilk production. Cyanobacteria are considered active agents in some speleogenesis processes. Northup et all. made evident in Lechuguilla cave an archaebacteria from *Crenarchaeota* group.

The diversity of the metabolic reactions explains the complex geo-chemical importance of such stones and shows that the extracellular organic compounds produced by the microorganisms have a big importance in connecting the biological processes with the geo-chemical ones.

These results bring up the possibility of using certain bacterial products (toxins) in medicine, and obtaining new antibiotics or other kinds of medicines for curing different diseases, including cancer.

Our aim in this study is to determine whether bacteria are present or not in the caves we have collected samples from: Ungurului Cave, Izvor Cave, Lesianei Cave, and Moanei Cave, from the Pădurea Craiului Mountains, and if there are any enzymatic activities mainly due to the microorganisms.

Matherials and Methods

Sampling. For microbiological analysis, samples were taken in aseptic conditions from different places of the four caves: floor deposits, wall deposits and sludge. The only cave which misses the sludge sample is the Lesianei Cave, and this due to the fact that there was no accessible water stream. Sampling took place in April, the 1st , September, the 9th , 2005 and January, the 17th 2006.

The enzymatic activities we have studied were:

Catalase activity was determined using a technique based on KAPPEN's method (1993). We took 1 g of material, added 10 ml of distilled water and 2 ml of H_2O_2 3 %. This mixture was incubated at 20 ºC, for one hour. The catalase activity is calculated from the difference between the active samples and the inactivated ones, and is expressed in mg H_2O_2/1 g material/5 h/20 ºC.

Dehydrogenase activity was determined using CASIDA et al. method (1964). The mixture consisted of 1 g of material, 0.5 ml TTC 3 % and 2 ml distilled water, for the actual activity. For the potential activity, instead of 2 ml of water we used only one and added another ml of glucose 3 %. Incubation took place at 37 ºC, for 72 h. The activity was measured in mg formazan/1 g material/72 h/37ºC.

Phosphatase activity was determined using the method of Kramer and Erdely, by using a mixture consisting of 1 g of material and 10 ml of disodic fenilphosphate solution 0.5 %. For incubation took more than 2 h, we added 2 ml of benzene. Incubation took place at 37 ºC, for 3 days. After incubation, we added ammonium alaun solution, borax tampon (pH 9.4) and Gibbs reagent. The intensity of the blue color shows the intensity of the reaction. Phosphatase activity was measured in mg phenol/1 g material/72 h/37ºC.

Urease activity was determined using Drăgan-Bularda's method (2000), the principle of which consists of mixing of the sludge samples with a urea solution, extracting and determining the liberated ammonia.

Non-enzymatic catalytic activity was established also using KAPPEN's method: 1 g material, 2 ml H_2O_2 3 %, 10 ml distilled water, but in this case samples underwent a thermic inactivation. Non-enzymatic catalytic activity also expressed in mg H_2O_2/g material/5 h/20ºC.

We have also computed the EISQ – which is the enzymatic indicator of soil or sludge quality – based on the results obtained by studying the enzymatic activities.

Results

a) Catalase and non-enzymatic catalytic activities.

Table no. 1 shows that the maximum values of the enzymatic catalase activity were detected in the samples collected from the floor deposited of the Izvor Cave, in all three sampling campaigns, so as: 4,21 mg H_2O_2 / g material (April, 2005), 4,17 mg H_2O_2 / g material (September, 2005) and 4,23 mg H_2O_2 / g material (January, 2006). The lowest values of the catalase activity were detected also in Izvor Cave, only this time in the wall deposits: 0,90 mg H_2O_2 / g material (April, 2005), 0,95 mg H_2O_2 / g material (September, 2005 and January, 2006).

Table no. 1. Enzymatic catalase activity and non-enzymatic catalytic activity

Sample origin	Sample	Catalase activity (mg H_2O_2 / g material / 5 h / 20 ºC)				Non-enzymatic catalytic activity (mg H_2O_2 / g material / 5 h / 20 ºC)			
		Campaign 1	Campaign 2	Campaign 3	Average	Campaign 1	Campaign 2	Campaign 3	Average
Ungurului Cave	Floor	2.38	2.29	2.33	2.33	1.03	1.11	1.10	1.08
	Wall	2.43	2.5	2.39	2.44	1.51	1.5	1.41	1.47
	Sediments	2.45	2.41	2.37	2.41	1.49	1.42	1.3	1.4
Izvor Cave	Floor	4.21	4.17	4.23	4.2	0.03	0.02	0.02	0.02
	Wall	0.9	0.95	0.95	0.93	0.09	0.08	0.1	0.09
	Sediments	2.31	2.37	2.4	2.36	0.51	0.47	0.41	0.46
Lesianei Cave	Floor	2.3	1.73	1.8	1.94	2.7	2.11	1.9	2.23
	Wall	1.19	1.23	1.11	1.17	1.9	1.78	1.87	1.85
Moanei Cave	Floor	2.45	2.37	2.31	2.37	3.17	3.2	3.16	3.17
	Wall	1.07	1.09	1.09	1.09	0.98	0.89	1.02	0.96
	Sediments	1.75	1.68	1.8	1.74	2.05	0.07	2.2	2.1

Table no. 2 . Total catalytic activity

Sample origin	Sample	Total catalytic activity (mg H_2O_2 / g material / 5 h / 20 ºC)			Average
		Campaign 1	Campaign 2	Campaign 3	
Ungurului Cave	Floor	3.41	3.4	3.43	3.41
	Wall	3.94	4	3.8	3.91
	Sediments	3.94	3.83	3.67	3.81
Izvor Cave	Floor	4.24	4.19	4.25	4.22
	Wall	0.99	1.03	1.05	1.02
	Sediments	2.82	2.84	2.81	2.82
Lesianei Cave	Floor	5	3.84	3.7	4.18
	Wall	3.09	3.01	2.98	3.02
Moanei Cave	Floor	5.62	5.57	5.47	5.55
	Wall	2.05	1.98	2.11	2.04
	Sediments	3.8	3.75	4	3.85

The same table indicates that as far as the non-enzymatic catalytic activity is concerned, the highest values were detected in the floor samples collected from Moanei Cave, in all the three sampling campaigns, as follows: 3,17 mg H_2O_2 / g material (April, 2005), 3,20 mg H_2O_2 / g material (September, 2005) and 3,16 mg H_2O_2 / g material (January, 2006). The lowest non-enzymatic catalytic activity values were registered in the case of the floor deposits of Izvor Cave, in all sampling campaigns, these values being: 0,03 mg H_2O_2 / g material (April, 2005), 0,02 mg H_2O_2 / g material (September, 2005 and January, 2006).

In most of the cases, samples show quite high value oscillations as far as the enzymatic catalase activity is concerned, especially according to the nature of the studied material. The only cave which showed a steadiness in this particular aspect is the Ungurului Cave, which exhibits rather constant values of the catalase activity both from the studied material point of view and sampling period. The rest of the caves indicated values of the catalase activity of the wall samples 2-4 times lower than samples from the floor deposits and sediments.

The same thing can be said regarding the non-enzymatic catalytic activity. Thus, samples exhibit diverse values, according to the: sampling period, cave, and the studied material. The steadiest cave from this point of view was again Ungurului Cave.

b) Actual and potential dehygrogenase activity.

The highest values recorded in the actual dehydrogenase activity were in the floor samples of Moanei Cave, in all the three sampling campaigns: 0,110 mg formazan/1 g material in April 2005; 0,109 mg formazan/1 g material in September, 2005; and 0,117 mg formazan/1 g material in January, 2006.

The lowest values recorded in the actual dehydrogenase activity were in the floor samples of Lesianei Cave, in September, 2005 (0,018 mg formazan/1 g material) and January, 2006 (0,013 mg formazan/1 g material) and sediments samples from Moanei Cave also in September, 2005 (0,011 mg formazan/1 g material) and January, 2006 (0,019 mg formazan/1 g material).

Mostly, both actual and potential dehydrogenase activities values maintain themselves steady during the period the study was made, except Lesianei Cave floor samples and Moanei Cave sediments samples, in which cases both the actual and the potential dehydrogenase activities exhibit a decrease in intensity during the second and the third campaigns, in comparison with the April, 2005 campaign.

c)Phosphatase activity.

Floor samples belonging to the Izvor Cave registered the highest values as far as the phosphatase activity is concerned, in all the three sampling campaigns: 0,311 mg phenol/1 g material (April, 2005), 0,302 mg phenol/1 g material (September, 2005) and 0,309 mg phenol/1 g material (January, 2006). The lowest value could be detected also in the Izvor Cave, in the wall sample from the first campaign, in April, 0,07 mg phenol/1 g material.

As a main characteristic, one can observe quite a high degree of oscillation of the phosphatase values, mainly according to the nature of the studied material. Thus, wall samples exhibit the lowest phosphatase activity values, 2-4 times lower than the floor or sediment samples, in all the caves we have taken into study. This can only demonstrate that the floor deposits and the sediments are the main source of phosphor in the cave environment.

Table no. 3. Actual and potential dehygrogenase activity.

Sample origin	**Sample**	**Actual dehydrogenase activity (mg formazan/1 g material/72h /37ºC)**				**Potential dehydrogenase activity (mg formazan/1 g material/72h /37ºC)**			
		Campaign 1	Campaign 2	Campaign 3	Average	Campaign 1	Campaign 2	Campaign 3	Average
Ungurului Cave	Floor	0.065	0.071	0.069	0.068	0.109	0.112	0.117	0.112
	Wall	0.091	0.093	0.092	0.092	0.200	0.210	0.201	0.302
	Sediments	0.036	0.029	0.039	0.034	0.063	0.057	0.08	0.066
Izvor Cave	Floor	0.095	0.098	0.098	0.097	0.405	0.411	0.401	0.405
	Wall	0.023	0.026	0.022	0.023	0.105	0.093	0.098	0.098
	Sediments	0.085	0.084	0.081	0.083	0.386	0.381	0.380	0.382
Lesianei Cave	Floor	0.058	0.018	0.013	0.029	0.315	0.082	0.071	0.156
	Wall	0.079	0.072	0.077	0.076	0.185	0.191	0.183	0.186
Moanei Cave	Floor	0.11	0.109	0.117	0.112	0.310	0.300	0.303	0.304
	Wall	0.055	0.049	0.059	0.054	0.099	0.107	0.104	0.103
	Sediments	0.091	0.011	0.019	0.040	0.277	0.072	0.079	0.142

Table no. 4. Phosphatase activity.

Sample origin	Sample	Phosphatase activity (mg phenol/1 g material/72 h/37ºC)			
		Campaign 1	Campaign 2	Campaign 3	Average
Ungurului Cave	Floor	0.223	0.263	0.249	0.245
	Wall	0.097	0.08	0.091	0.089
	Sediments	0.197	0.200	0.087	0.161
Izvor Cave	Floor	0.311	0.302	0.309	0.307
	Wall	0.07	0.081	0.084	0.078
	Sediments	0.235	0.229	0.23	0.231
Lesianei Cave	Floor	0.21	0.207	0.289	0.235
	Wall	0.115	0.123	0.121	0.119
Moanei Cave	Floor	0.205	0.119	0.209	0.204
	Wall	0.09	0.085	0.079	0.084
	Sediments	0.119	0.140	0.136	0.131

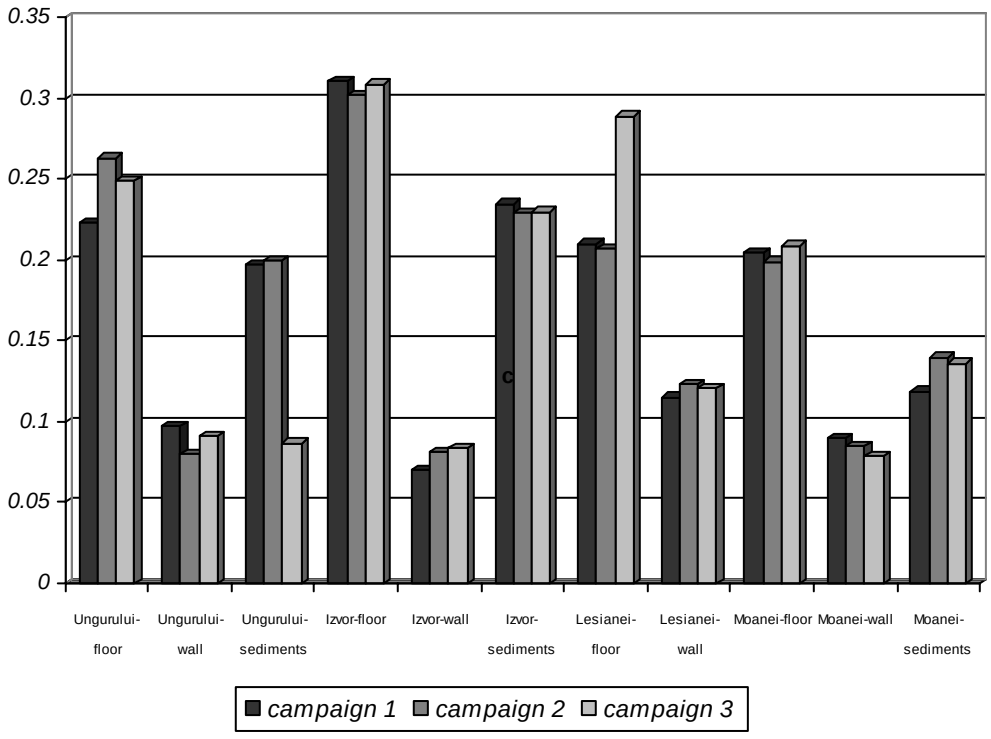

Fig. 1. Phosphatase activity

d) Ureasea activity.

As far as the ureasea activity is concerned, most of the times, the values registered were quite low, the highest ones being those from the Lesianei Cave, in all the three sampling campaigns, on the floor samples: 2,79 mg NH_4/1 g material (April, 2005), 2,77 mg NH_4/1 g material (September, 2005) and 2,85 mg NH4/1 g material (January, 2006). The lowest ureasea activity values appear in the sediment samples from the Moanei Cave: 0,63 mg NH_4/1 g material (September, 2005) and 0,55 mg NH_4/1 g material (January, 2006).

Most of the times, the urease activity values maintain themselves constant along the year, except the floor samples from Izvor cave, where the values from the campaigns in April, 2005 were low (1,97 mg NH_4/1 g material), January, 2006 (1,83 mg NH_4/1 g material) and a higher value in September, 2005 (2,47 mg NH_4/1 g material).

The values of the urease activity maintain themselves constant also according to the nature of the analyzed material, especially Ungurului and Izvor caves. As far as the Lesianei and Moanei Caves are concerned, the urease activity values in wall and sediment deposits were twice as low as those in the floor samples.

All the enzymatic activities which have been measured quantitatively (enzymatic catalase activity, non-enzymatic catalytic activity, actual and potential dehydrogenase activities, phosphatase activity and urease activity) were properly detected in all the three sampling campaigns and in all sampling sites.

Table no. 5. Urease activity.

Sample origin	Sample	Ureasea activity (mg NH_4 /1 g material)			
		Campaign 1	Campaign 2	Campaign 3	Average
Ungurului Cave	Floor	1.85	1.71	1.92	1.82
	Wall	1.63	1.55	1.48	1.55
	Sediments	1.54	1.61	1.53	1.56
Izvor Cave	Floor	1.97	2.47	1.83	2.09
	Wall	1.57	1.51	1.54	1.54
	Sediments	1.17	1.15	1.19	1.17
Lesianei Cave	Floor	2.79	2.77	2.85	2.80
	Wall	1.78	1.91	1.89	1.86
Moanei Cave	Floor	1.12	1.01	1.06	1.063
	Wall	0.77	0.81	0.82	0.80
	Sediments	0.89	0.63	0.55	0.69

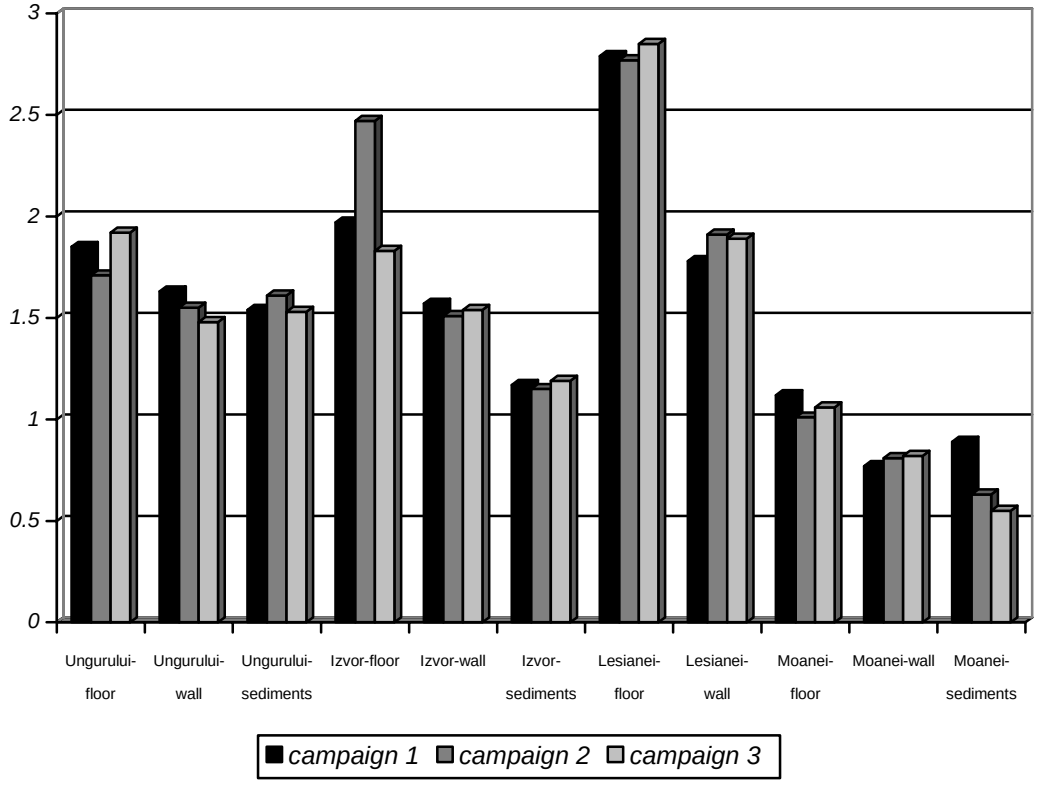

Fig. 2. Urease activity

The values of the studied activities varied mostly according to the cave taken into account, the origin of the sample (aquatic sediments, wall or floor deposits) but less according to the sampling campaign, thing we were actually expecting due to the fact that the cave environment maintains its conditions steady along the entire year.

The enzymatic indicator of soil or sludge quality - EISQ

The IESQ represents an index that shows the enzymatic potential of a soil or aquatic sediment, of a bacteria culture or even of water.

The higher the enzymatic potential, the more intense the microbial activity is, the more intense the decomposing processes are, while the organic matter turns into minerals more rapidly and with a higher efficiency. An environment exhibiting a high enzymatic potential is a fertile, non-polluted environment.

In computing IESQ one needs to measure several enzymatic activities. The more enzymatic activities are taken into account, the more precise the IESQ is.

From an enzymatic point of view, the quality of the studied sediments is characterized by the intensity of the enzymatic activities which is defined by the values of the enzymatic indicator of soil or sludge quality.

Theoretically, the values of the enzymatic indicator of soil or sludge quality can lie between 0 (when the sample exhibits no enzymatic activity) and 1 (when all the actual individual values equal the theoretical individual maximum values of each of the samples).

The highest values recorded in the case of the IESQ belonged to the samples from the floor deposits in the Izvor and Moanei Caves, as shown in fig. 3.

Conclusions

Microorganisms exist in all analyzed samples (floor and wall deposits, sludge), but in limited strength;

This analysis and the positive results of the enzymatic tests denote the presence of microorganisms in caves, both at the time being and in the past;

The values registered in the enzymatic activities studied in this work kept themselves constant during the entire year, with small exceptions, thing that we had expected as there are no significant fluctuations concerning the cave environmental conditions;

After computing the IESQ, it results that the highest values recorded belonged to the samples from the floor deposits in the Izvor and Moanei Caves, these caves exhibiting the highest enzymatic potential;

By studying the microbiota of the cave environment, more valuable discoveries can be made in the field of the microbe biotechnology.

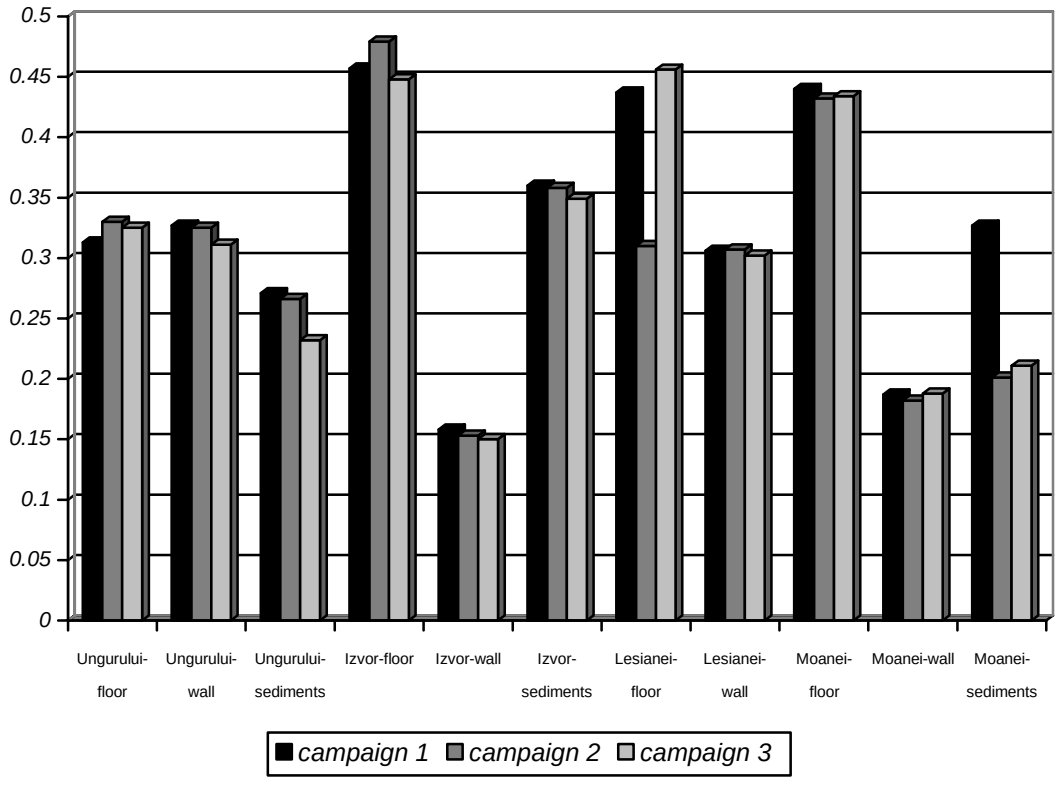

Fig 3. The values of the enzymatic indicator of soil or sludge quality - EISQ

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