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SOME PECULIARITIES OF COLLEMBOLAN DISTRIBUTION IN THE RIVERSIDE ZONES OF THE DNIESTER RIVER

GALINA BUSMACHIU[1] AND ELENA ZUBCOV[1]

SUMMARY. The comparative analysis of the state of soil along the banks of Dniester River using collembolan communities' structure was done. In spite of permanent floodings and transformations, the riverside sectors remain rather attractive places for the soil invertebrates, due to the carry-over and accumulation on their surface of the biogenic elements. It was established high species diversity of Collembola even when the number of individuals was low and the correlation of their density with the soil organic matter and water content.

Keywords: bank of river, Collembola, ecology

Introduction

The ecological researches of the river banks and floodplains using small groups of invertebrates present special interest in our days (Khanislamova, 1988; Bulimar, 1992; Čarnogurský, 1998; Sterzynska and Ehrnsberger, 1999; Sterzynska and Pilipiuk, 1999; Russell *et al*., 2004; Tronstad *et al*., 2005; Busmachiu, 2004 and 2006). The bank of rivers and floodplains are in a permanent transformation. During the spring river floods or after abundant rains the level of river water increase greatly and transcend the usual bank limit, bringing to the banks biogenic elements such as fine particulate organic matter (Junk, Bayley and Sparks, 1989). It was found that the soil invertebrates' communities living in the margin on aquatic basins are able to cope with highly variable wet and dry condition depending on the frequency and duration of inundation. Collembola are one of the most significant and important studied groups that can be find in the soil and sand of riverbanks, on wetland, on aquatic vegetation, on the decomposing organic matters, which have an active role in matter cycling and energy transformation in nature. The Collembola remain one of the less studied group of river ecosystems, although it is an important connecting group within the trophic chains. The diagnosis of riverbank status, using soil zoology studies, represents one of the control methods of environment ecological conditions as a whole (Van Straalen, 1991). The life of small invertebrate organisms depends on environmental condition; they quickly respond to habitat disturbances due to their high sensitivity. The structure of collembolan communities, the presence or absence in different proportions of living forms and

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some bioindicator species reflect the state of aquatic basin. In present the physico-chemical studies of the quality of fresh water are not enough, we must take also into consideration the state of aquatic basin in general.

As the result of our previous research (Busmachiu, 2004),carried out in different types of biotopes adjacent to the Dnister River banks, 42 species of Collembola, belonging to 28 genera and 10 families, were recorded.

This paper presents the results of ecological studies accomplished straight on the water/soil gradient of the Dniester bank, exposed to periodical flooding. The aim of this research is to investigate the collembolan distribution along the river stream and gradually up to 8 m from the bank line, as well as the influence of soil parameters upon the structure of collembolan communities.

Materials and Methods

Characteristics of studied localities. Dniester is the biggest river in Moldova, which provides the main water resources in the republic. The river length along the territory of Moldova is of 657 km, the water basin surface constitutes 19.1 thousand km^2, which represent about 70% of the republic territory and the velocity of river stream is of 0.5 m/sec. The Dniester riverbed is sinuous in the upper course; it penetrates the calcareous formations of toltre reefs that emerge to the ground surface as cliffs and rocky banks. The middle Dniester banks are argilo-arenaceous and sand-slimy.

- Naslavcea village (48^0 29'21" N, 27^0 34' 49" E) is situated at the altitude of 209 m. The water level in the river, during the sample collection, was raised; the bank was partially inundated, covered by herbaceous plants. Furthermore, the rocks (granite) emerge on the surface and the bank is covered here and there by stones and crushed rock.
- Otaci village (48^0 26'49" N, 27^0 41' 01" E) is situated at the altitude of 63 m. The bank was smooth, partially flooded, with abundant herbaceous vegetation.
- Soroca town (48^0 $08^{'}$41" N, 28^0 $17^{'}$ $44^{"}$ E) is situated at the altitude of 42 m. The water level in the river was raised, the bank was partially inundated, roughly covered by debris of decomposing vegetation and the soil is sludgy. The bank line was indistinct.
- Camenca town (48^0 $00^{'}$50" N, 28^0 $41^{'}$ $51^{"}$ E) is situated at the altitude of 33 m. The water level in the river was raised; the bank was partially inundated, covered by herbaceous plants.
- Goieni village (47^0 $22^{'}$ 20" N, 29^0 $08^{'}$ $57^{"}$ E) is situated at the altitude of 18 m. The water level in the river was raised; the bank was partially inundated, covered by abundant partially decomposed plants and herbaceous vegetation.

The soil temperature was measured at the moment of the sampling; the percentage of water and organic matter content in soil, taken linearly at the distance of 0-2 (I line), 2-4 (II line), 4-6 (III line) and 6-8 (IV line) meters from water edge, as shown in table 1, were determined in laboratory.

Table 1.

The main soil parameters in the studied localities

Localities	Soil parameters								
		Organic matter, %				Water content ,%			
	T^0C of soil	Collection line and distance from bank, in meters							
		I 0-2m	II 2-4m	III 4-6m	IV 6-8m	I 0-2m	II 2-4m	III 4-6m	IV 6-8m
Naslavcea	16.0	9.44	6.92	7.54	5.76	28.92	15.85	14.22	11.05
Otaci	16.2	13.12	12.14	11.57	10.79	29.50	26.91	20.14	10.47
Soroca	13.6	16.77	12.44	15.43	13.21	33.40	24.96	28.52	20.39
Camenca	15.2	14.04	12.68	11.19	12.15	31.39	21.06	19.76	17.12
Goieni	16.1	14.19	14.53	16.60	14.91	27.11	27.02	27.32	16.40

Sampling and extraction. The faunistic material was sampled along the banks of the Dniester River, in the above mentioned sites, at the beginning of May, 2006. The samples were taken from the northern Naslavcea village to Camenca town and Goieni village, where the Dubasari water reservoir begins.

At each locality an area of 8 x 8 m was selected, starting from the water edge and divided into 16 sampling points with 2 x 2 m size. More than 80 soil samples, taken by a square frame with surfaces of 25 cm^2 and 5 cm depth, were collected. The microarthropods were extracted from the soil using the flotation method. The specimens of Collembola were fixed in 80^0 ethylalcohol and identified up to the species level according to keys of Stach (1947-1963), Gisin (1960), Pomorski (1998), Bretfeld (1999), Potapov (2001) and other systematics papers.

Margaleff (Im), Shannon (Ish) and evenness (Magurran, 1991) indices were calculated for the estimation of the diversity of collembolan communities in the studied localities.

The soil analyses were done using methods generally accepted in soil science (Arinushkina, 1961); the statistical processing was realized using EXCEL program.

Results and Discussion

As a result of investigation, 31 species of Collembola belonging to 23 genera and 10 families were found and 1889 collembolan individuals were registered in the studied localities along the Dniester River. Their density varied between a minimum of 5,825 ind.m^{-2} near Camenca town and a maximum of 15,700 ind.m^{-2} near Goieni village on the bank of the aquatic reservoir (Table 2). The minimum of collembolan density and species diversity were recorded at the distance up to 2 m from the water edge in all studied localities. The total number of registered species per site was approximately the same, 15 – 16 species, but near Naslavcea village only 11 species were found. The minimum number of individuals (20) was found up to 2 m distance from the water edge near Naslavcea village and maximum number (267) up to 4 m from the water edge near Camenca town.

Table 2.

Comparative analysis of collembolan community structure from the soil along the bank of Dniester River

Localities	Density ind.m^{-2}			Total species	Indexes		
	Mean dens. ± SD	Min	Max		Ish	Im	Even-ness
Naslavcea	6,840 ± 4,86	2,000	12,200	11	2.46	1.78	0.71
Otaci	9,350 ± 4,20	5,400	15,300	15	2.59	2.19	0.68
Soroca	10,500 ± 3,82	6,900	15,400	16	2.43	2.48	0.60
Camenca	5,825 ± 3,91	2,400	10,100	16	2.28	2.32	0.57
Goieni	15,700 ± 7,22	5,300	21,400	16	2.86	2.75	0.71

In the study of the comparative structure of collembolan communities, it is very important to take into account the main soil parameters, such as water content, temperature and organic matter content. These parameters represent the main factors of influence upon the number and species diversity of small soil invertebrates. As the data collecting took place at the beginning of May only, there were not any significant differences between the temperature characteristics, but the parameters of soil water content and the organic matter content varied. The mentioned parameters were significantly distinguished not only in different sampled localities, but also at the different distance from the water edge. The main soil parameters were considered linearly up to 8 m towards the river water edge.

The data on the soil water content and the organic matter content emphasized a rather distinctive peculiarity. In all studied localities, the maximum soil water content was recorded on the water/soil gradient and up to 2 m from the water edge: from 33.40% in Soroca to 26.91% in Otaci where the lowest values of collembolan individuals and species diversity were registered. Moving away from the water edge the soil water content decreased gradually, but the collembolan density and species diversity increased. At the distance of 8 m, the longest distance from the water edge, the soil water content had the minimum values from 11.05% in Naslavcea to 10.47 % in Otaci and the species diversity was great.

The gradual decreasing of soil water content observed in the majority of localities was recorded only on smooth, gradually raising bank line. However, near Soroca, the bank line was rough and low; respectively the parameters of humidity varied on the entire studied sector, including the last fourth line up to the distance of 8 m from the water edge (Table1). The same irregularity was registered in the distribution of collembolan number. Two high values up to 4 m (116 ind.) and up to 8 m (154 ind.) from the river water edge were recorded. In other areas, namely near Goieni and Otaci, only one maximum number of individuals was recorded up to 6 m (214 ind. and 153 ind., respectively); near Naslavcea and Camenca, up to 8 m,

99 and respectively 101 individuals were recorded. The soil water content differences between the first (up to 2 m) and the last lines (up to 8 m) are considerable and fluctuated from 19.03 % in Otaci, 17.87 % in Naslavcea, 14.17 % in Camenca, 13.01% in Soroca and 10.71% in Goieni. Thus, the linear material collection allows to emphasize the linear modification of soil water content, as well as of collembolan number and species diversity distribution at various distances from the water edge.

The content of the organic matter in the soil have also the tendency to decrease as moving away from the water edge. Its maximum quantity was registered in the first lines 16.77% in Soroca and 14.19% in Goieni, while the minimum quantity was registered in the fourth lines 5.76% in Naslavcea and 10.79% in Otaci. The edge of the bank river near Goieni was an exception. Here, at the distance of 4 - 6 m from the water edge, the decomposing algae aggregations were noted and, as a result, a high level of organic matter content– up to 16.60% and the highest value of collembolan number (214 individuals) were recorded.

The greatest collembolan density on square meter was recorded near Soroca town and Goieni village, where the highest level of organic matter content in the soil was noted. Here the organic matter content in the water is also increased.

A positive significance was emphasized between the organic matter content of the soil and the collembolan density in the studied sectors (Fig.1).

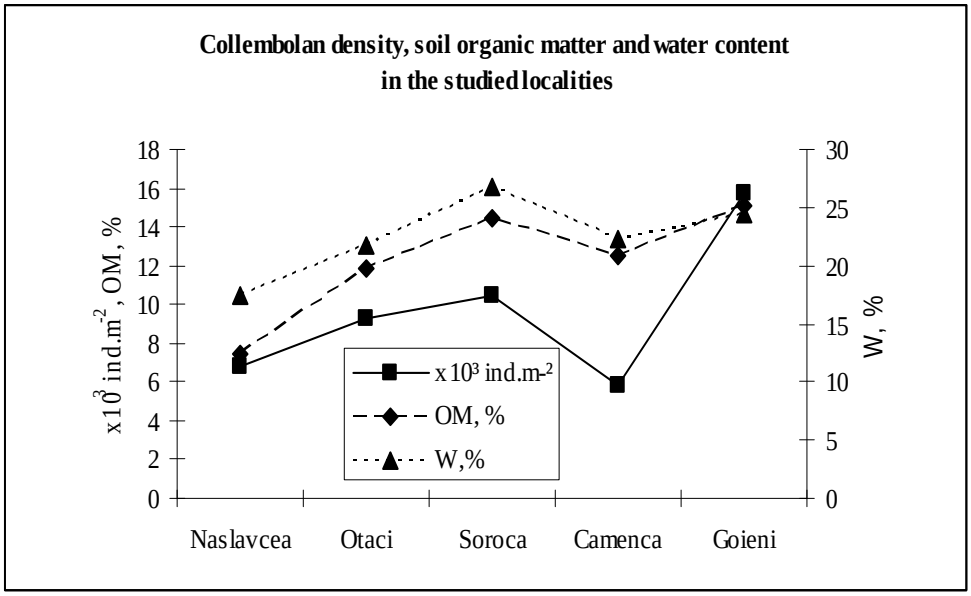

Fig.1. The average density of Collembola (x 10^3 ind.m^{-2}), soil organic matter (OM %) and water content (W %) in the studied localities

The study of collembolan species diversity and density at the distances up to 0 - 2, 2 - 4, 4 - 6 and 6 – 8 meters from the water edge allows to emphasize several peculiarities of the species distribution. Thus, at the distance up to 2 m an average of 4 – 5 species were present in number of 20 to 125 individuals. Among them the hydrophilous species such as *Cryptopygus thermophilus*, *Anurida ellipsoides* and *Folsomia candida* were dominant. Moving away from the water edge of river, the collembolan species composition changed. Along with the soil water content decreasing the number of small deep-soil species, as well as of large ecological spectrum species increased, such as *Mesaphorura krausbaueri*, *Protaphorura serbica*, *Folsomides parvulus*, *Orthonychiurus rectopapillatus*, *Isotomodes productus*, *Lepidocyrtus paradoxus* and *Sminthurinus elegans.*

Our results correspond to those of other researches concerning the development of collembolan communities in areas of increased soil humidity, but not flooded by water. Sterzynska and Pilipiuk (1999) found the highest collembolan number and species diversity in such zones situated at the distance of 4-8 m from the water edge.

The soil up to 6 m from the river water edge was the most populated (abundance), while the highest species number, including large athmobionts, characterized the soil up to 8 m. Here, the following species such as *Neanura muscorum*, *Orchesella albofasciata*, *Orchesella multifasciata*, *Orchesella xerothermica*, *Pseudosinella horaki*, *Heteromurus major* and *Sminthurinus bimaculatus* were found.

The species *Cryptopygus thermophilus*, which has an eurytopic distribution and is considered as a thermophilous and nitrophilous species (Potapov, 2001), was the dominant taxon on the bank near Camenca town. Often this species has a high number of individuals in the industrially polluted soils, including soils with increased content of heavy metals.

In Moldova, a high density of this species was observed, mostly in compost and dunghills (unpublished personal observation). It was also found dominating up to 57.15% of the entire collembolan communities near Giurgiulesti village on Prut River bank, at the confluence of Prut with the Danube (Busmachiu, 2006). This fact represents a distinctiveness of unstable, disturbed ecosystems (Van Straalen, 1991).

The highest values of Shannon diversity index – 2.86, of Margaleff species abundance index – 2.75, as well as of evenness index – 0.71 were registered near Goieni village (Table 2), in contrast to Soroca town, where a high density of collembolan population was recorded, while the mentioned indexes values were comparatively low.

Conclusions

The comparative analysis of the state of soil along the banks of Dniester using collembolan communities' structure of five localities emphasized several peculiarities of collembolan species diversity and distribution in riverside zones. In spite of permanent floodings and transformations, the riverside sectors remain rather attractive places for the soil invertebrates, due to the carry-over and accumulation on their surface of the biogenic elements. Thereby, Collembola

inhabited all the studied localities from the bank of Dniester River and their species diversity and density are varying and correlate with the soil water content and organic matter content.

The linear sample collection allow to emphasize the gradually decreasing of water content and organic matter content up to minimum values at the distance up to 8 m from the edge of river bank and vice-versa, the increasing of collembolan number and species diversity up to maximum values at the distance of 6-8 m from the water river edge. It is important to mention a rather high species diversity of Collembola, even when the number of individuals was low. In spite of sharp water level fluctuations in the river, in the soil on the riverbank though remain deep-soil collembolan species that are most important for the soil genesis processes.

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ON THE ANT EATING SPIDERS (ARANEAE: ZODARIIDAE) OF ROMANIA: NEW FAUNISTICAL DATA

IOAN DUMA[1]

SUMMARY. Although Romania has 5 biogeographic regions, more than in any neighboring country, till 2006 were recorded only four *Zodarion* species. This study reveals the existence of *Zodarion morosum* Denis 1935 and *Zodarion cyprium* Kulczynski 1908 in Romanian fauna. The biogeography of the Romanian species of *Zodarion* is discussed and notes of their habitat are given.

Keywords: Arachnida, *Zodariidae*, *Zodarion cyprium*, *Zodarion morosum*, habitats, Romania

Introduction

From a total of eleven biogeographic regions found in Europe according to the European Environment Agency, in Romania five are present: continental, alpine, pannonian, pontic and steppic, more than in any other country on the old continent (except Russia). So we would expect to find here a great biodiversity of flora and fauna.

In spite of this potential till 2006 there were only four species of *Zodarion* recorded in our fauna Weiss and Petrişor (1999). From these *Zodarion geticum* Weiss, 1987 and *Zodarion aurorae* Weiss, 1982 were described for the first time from Romania by Weiss (1982), (1987). Recently Duma (2007) added to the list *Zodarion rubidum* Simon, 1914 but still this number is very small in comparison to the species known from the neighboring countries: Bulgaria has 11 recorded species according to Deltshev (2005), Macedonia (part of the former Yugoslavia) – 10 species, Blagoev (2002), Ukraine - 5 species, Kovblyuk (2003).

This paper adds further two species of the genus *Zodarion* for the Romania: *Zodarion cyprium* Kulczynski 1908 and *Zodarion morosum* Denis 1935.

Also the biogeography of all *Zodarion* species known from Romania is discussed and notes on their habitat are presented.

Material and Method

The spiders were collected in the summers (July) of 2004 and 2007 from South-Eastern Romania (Dobrogea and Muntenia regions) and then preserved in ethylic alcohol 70%. All specimens of *Zodarion* were gathered by hand from the vicinity of ant trails or nests.

For the determination of the material we used online papers of Nentwig & all. (2003) and Weiss (1982), (1987).

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The geographical coordinates of the collection places were obtained with a Magellan and Yakumo Global Positioning System units or from the maps provided by Google Earth program.

The plant associations are according to the Habitats of Romania by Donita & all. (2005).

Abbreviations used in the figures.

Abbreviations used for the countries in the Balkan Peninsula:

RO – Romania, UKR – Ukraine, BG – Bulgaria, SRB – Serbia, MK – Macedonia, GR –Greece, T – Turkey, AB – Albania, CR – Croatia, MTN– Monte Negro, BOS – Bosnia and Herzegovina, SL – Slovenia.

Abbreviations used to specify the biogeographical distribution of the Zodarion species found in Romania.

EUR – Species with European distribution, BLK – species from Balkan area, P-MED – Ponto-Mediterranean species, STP – species with east European distribution found in Steppic regions.

Material deposition

All specimens collected are now deposited in the collection of Department of Biology, Faculty of Chemistry-Biology-Geography, West University of Timişoara.

Results

The spiders collected were identified as:

Zodarion cyprium Kulczynski 1908: 1♂ (7 July 2004, Agigea - 44º04′58″N 28º38′28″ E) and 1♀ (13 July 2007, Histria - 44º32′55″N 28º46′09″E).

Zodarion morosum Denis 1935 2♂♂ (4 July 2007, Eforie Nord - 44º03′39″N 28º38′20″E) 2♀♀ (one on 5 July 2007 at Agigea -44º05′24″N 28º38′31″E and one on 15 July 2007 in 2 Mai - 43º46′49″N 28º34′24″E) (fig. 1).

Zodarion geticum Weiss 1987 1♂ (27 August 2007, Bujoru 43º42′48″N 25º33′25″E) (these coordinates were taken from the Google earth program).

Zodarion cyprium Kulczynski 1908 and *Zodarion morosum* Denis 1935 were found on marine sand dunes along the Black Sea coast (fig. 1) in habitats with *Atripliceto hastatae - Cakiletum euxinae* Sanda & Popescu 1999, *Ephedro - Caricetu colchicae* (Prodan 1939 n.n. Morariu, 1959) Sanda & Popescu 1973, *Secali sylvestri - Alyssetum borzeani* (Borza 1931) Morariu 1959, *Schoenetum nigricans* W. Koch 1926, Doniță & all. (2005). Unfortunately these habitats are under constant human pressure and their surface is diminishing year after year due to the development of tourist resorts especially in the southern part of Romanian Black Sea coast.

With our new findings the total number of *Zodarion* spiders known in Romania rises to seven species.

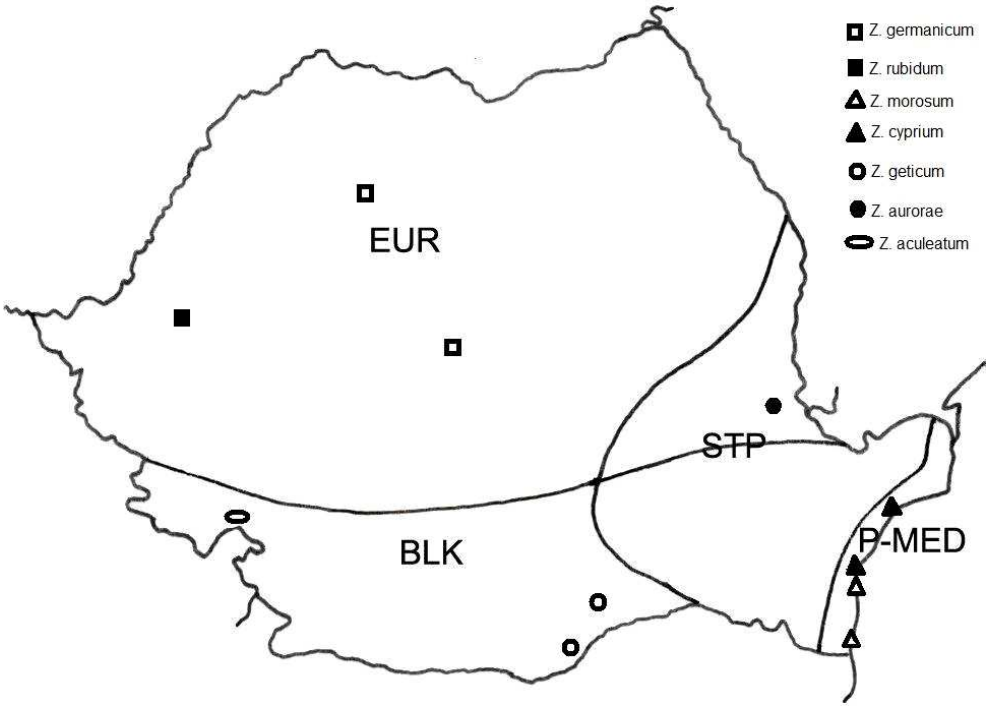

Figure 1. The distribution of *Zodarion* species in Romania

Zodarion geticum Weiss 1987 described from material gathered near capital Bucureşti by Dumitrescu is another rare species found in our searches (fig. 1). Till now it was recorded in Romania only from the Ceagău forest (Comana Natural Park) by Weiss (1987). From the moment it was described, no other report on this species has been published till present study. Unfortunately like Dumitrescu in 1987 we have collected just a male specimen and so the female remains still unknown to science. Present article adds a new distribution point for this rare species in Romania and brings new data on the habitat of the species. If Dumitrescu found his specimens in Moesian silver lime woods (Natura 2000) with the following plant associations: *Ornithogalo-Tilio-Quercetum* A. Dihoru 1976, *Quercetum pedunculiflorae* Borza 1937, *Querco pedunculiflorae-Tilietum tomentosae* Doniță 1970 we have found it in an orchard near the town of Alexandria at Bujoru (Teleorman district).

Discussions

The spiders of the Zodariidae family belong to a group of a few specialized arachnids that feed on ants. Their specialization seems to be so complex that at least some species don't grow well on any ant diet as shown by Pekar (2004). This

may lead to the conclusion that the dispersal of a *Zodarion* species is conditioned by the presence of certain species of ants. However in the end we believe that both ants and spiders have their distribution conditioned by the climate and habitat conditions and in this context we shall discuss the distribution of the ant eating spiders in Romania.

The presence of *Z. cyprium* and *Z. morosum* in Romania shows that the South-Eastern Romania has strong Mediterranean influences being home of many rare species for the country.

Although with present study *Z. geticum* has one more distribution point on the map of Romania we still know little about this species and the female remains still unknown to the science.

In 2001 it seems that was found also in Bulgaria Tzonev & Lazarov (2001) in the Osogovo Mountain (south-Western Bulgaria) at 950 meters altitude in a glade but this findings are not recognized by Platnick (2007) who still considers *Z. geticum* as a species limited to the Romanian territory.

Zodarion aurorae Weiss 1982 described by Weiss (1982) after material collected at Hanu Conachi reserve (fig. 1), in Galati district, on 5 May – 18 June 1977 by Marcu Aurora & Weiss (1979) remains the rarest species from the Zodariidae family in Romania.

The habitat of this species are sand dunes with *Brometum tectori* Bojko 1934, *Plantaginetum arenariae* (Buia et. al. 1960) Popescu Sanda 1987, *Mollugietum cervianae* Borza 1963, *Festucetum beckeri* Popescu et Sanda 1997. From its description till now it has never been recorded again by any arachnologist from Romania or abroad. It is the only species that has a present known distribution restricted to the southern Moldova region and till other faunistical data remains a Romanian endemic species. However judging the climate conditions from Hanu Conachi (medium annual rainfall between 500 and 400mm, average sunny days per year between 150-170 days, average temperature in January of -4ºC), and habitats in which *Z. aurorae* was found it can be inferred that it is a Steppic element and should be present and in nearby Ukraine.

Zodarion germanicum C.L. Koch 1837 seems to be the most common species in Romania (fig. 1) being found in Weiss's collection from Brukenthal Museum in Sibiu, Weiss (1976), in Fuhn's collection deposited now in the National History Museum "Grigore Antipa" from Bucuresti and also in private collections of other arachnologists: Urák (2002). It is however restricted to those regions that have continental influences (Western, Central and Northern Romania).

Zodarion rubidum Simon 1914 found till now just in the Banat region (fig. 1) of Romania by Duma (2007) may be a common and widespread species in all western and northern parts of the country especially along rivers. Our affirmation is based on its European distribution.

Zodarion aculeatum Kulczynski 1897 is a species restricted to the southern Romania being found till this day only by Fuhn according to the spider checklist

made by Weiss & Petrisor (1999) (fig. 1). This species seem to be restricted to the northern Balkan Peninsula being cited in the faunas of Serbia, Macedonia, Bulgaria and of course Romania.

From the biogeographical point of view Romania has a great faunistical potential. Its position in the northern Balkan Peninsula and the various climate influences has great repercussions on the fauna (fig. 2). In the western and northern regions where strong continental influences are present, European species are found: *Z. germanicum* and *Z. rubidum*. Further other widespread species may also be present.

The South-Eastern Romania has the greatest *Zodarion* diversity. Here are found ponto-mediteraneean species along the Black Sea coast: *Z. cyprium* and *Z. morosum*, then Steppic species: *Z. aurorae* and also Balkanic ones: *Z. aculeatum* and *Z. geticum*.

Conclusions

1. The total number of *Zodarion* species known from Romania until present is of seven.

2. Analyzing the geographical distribution of *Zodarion* species from our country it can be inferred that the greatest diversity of species is found in the South-Eastern Romania where are present ponto-mediteranean, steppic and balkanic species.

3. *Zodarion cyprium* and *Zodarion morosum* are ponto-mediteraneean species and therefore restricted to the Dobrogea region.

4. *Zodarion aurorae*, remains the rarest species from the genus *Zodarion* in Romania. Although it was found in a single place: Hanu Conachi reserve (Southern Moldova) it is a Steppic element and might be present in Ukraine also.

5. *Zodarion aculeatum* and *Zodarion geticum* are Balkan species with the northernmost distribution in Romania.

6. Due to the rarity of the species: *Zodarion geticum* and *Zodarion aurorae* and also because their habitat is under constant pressure we suggest that these species should have a place on a red list of Romanian spiders that must be done in near future.

7. Although with recent findings the number of *Zodarion* species rose to seven each of these has just a few known distribution points and so further faunistical studies on this family are needed for clarifying their status.

Figure 2. The position of Romania in the Balkan Peninsula and the influences on the *Zodarion* spider fauna

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POPULATION FLUCTUATIONS AND THE SPATIAL HABITAT USE BY AMPHIBIANS IN A HUMAN MODIFIED LANDSCAPE

TIBOR HARTEL[1,2] AND COSMIN IOAN MOGA[2]

SUMMARY. In this study we present the start of the breeding season and the long term population fluctuations of amphibians in a permanent pond and a number of temporary ponds in a human modified landscape in the middle section of the Târnava Mare basin. The start of breeding was strongly influenced by temperature in spring. The populations of *Hyla arborea* and *R. temporaria* were in decline whereas *Pelobates fuscus* and *Bufo bufo* were stable during this time. *Pelobates fuscus* is represented by a small population and is probably maintained by immigration from other areas. *Bufo bufo* and *P. fuscus* use only the permanent pond for reproduction. More egg masses of *R. temporaria* were found in the temporary ponds than in the permanent pond. The reproductive success was yearly observed in the temporary ponds but not in the permanent pond. We assume that the permanent pond is a source habitat for *B. bufo* and represents sink for *R. temporaria* whereas the temporary ponds from the forest represent source habitats for the last species. This and the previous studies on this community suggest that in this landscape, both the permanent and temporary ponds and the landscape connectivity are crucial for the maintenance of rich amphibian communities.

Keywords: amphibian populations, climate, long term fluctuations, landscape

Introduction

The human domination on the earth ecosystems substantially altered the Earth systems through several interacting processes (Vitousek *et al*., 1997). It was recognized that designing protected areas does not guarantee the protection of biodiversity because (i) the protected area covered is still too small (Hoekstra *et al.*, 2005), (ii) human interests are continuously growing (Liu *et al.*, 2001). It is increasingly recognized that there is a significant biodiversity outside of the protected areas that should be also properly managed and protected (Daily et al. 2001, „2010 Biodiversity Indicators Partnership" http://www.twentyten.net/target.htm).

Habitat and population based studies in Romania are more important now than ever. Firstly, with the recent EU adhesion new land use practices will be (quickly) adopted, and this will lead to drastic changes in the landscape structure. Secondly, we have (maybe the last) opportunity to study populations in clutural landscapes, thus, to get information about the ways at which human perturbations in the landscape represented by traditional land use practices affect the distribution and persistence of the natural populations. New developements in population /

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metapopulation theory, island biogeography and landscape ecology and their integration in ecology, represent useful modern insights about how hypoheses should be formulated, data gathered and interpreted to achieve this goal (Sandersen *et al*., 2002; Fischer *et al*., 2004; Manning *et al*., 2004; Fischer *et al*., 2006; Fischer and Lindenmayer, 2007; Lindenmayer *et al*., 2007; Schneider and Willig, 2007).

Pond breeding amphibians are important focal group for conservation for a number of reasons: (i) They have complex life cycles and need high spatial heterogeneity of the landscape to complete it. These life history traits makes them sensitive to habitat loss and fragmentation. (ii) They often are organized in metapopulations (Alford and Richards, 1991; but see Smith and Green, 2005). Loss of connectivity between local populations may expose them to genetic depletion, this making them susceptible for a number of other natural and anthropogenic stressors (Edenhamn *et al*., 2000). (iii) Amphibians are in global decline (Stuart *et al*., 2004) for various and often complexly interacting causes (Beebee and Griffiths, 2005).

Monitoring amphibian populations and the spatial use of the habitats provide information about the factors (natural and anthropogenic) causing long term fluctuations and their potential role as source populations at landscape level. In this paper we present the population fluctuation in 11 years of four amphibian species in the middle section of the Târnava Mare basin. The area is a mosaic patch that has natural – seminatural (ponds, river, grassland, forest) and anthropogenic (railway, arable lands, built areas) origins. The composition and configuration of the above mentioned landscape elements allow the study of the role of spatial arrangement of these landscape elements in determining the spatial extent of habitat use by amphibians. Previous studies have shown that the distribution of the migrating and dispersing Common Toads can be related to these land use types, the grassland between the pond and the forest being of critical importance (Hartel and Demeter, 2005; Hartel *upublished results*). In a larger scale, both the landscape composition and configuration was found to be important predictors of the Common Toad population sizes (Hartel and Moga, 2007 *in press*). Moreover, the long term study regarding the temporary habitat use by the Yellow Bellied Toad (a Habitat Directive species) in this area (Hartel *et al*., 2007a) shows that this species use the most durable ponds for reproduction in dry years. Temporary ponds that act as sink habitats in dry years represent high quality breeding habitats in rainy years. Because of the large and stable populations of some species the area was proposed as Natura 2000 Site of Community Importance (Ministerial Order 776/2007). Yet the whole area where the temporary ponds occur is seriously impacted by the drainage ditches that were made in order to facilitate gas extraction and the movement of heavy vehicles towards and from the gas source stations (2007).

In this study we present:

a) aspects of the breeding phenology (start of breeding season) in two species,
b) the long term fluctuations in the amphibian populations,

c) the correlation of the start of breeding period and population fluctuations with a number of climatic variables.

Materials and Methods

Study area. The study area is situated in the middle section of the Târnava Mare Valley, Romania (46º13'47.8''N; 24º46'47.6''E, 345 m altitude) and has approximately 3 km² area. A permanent pond and up to 80 temporary ponds were studied in this area (Fig. 1).

The permanent pond has 2.2 ha area with a maximum depth of about 4 m. The reed cover in the pond shows gradual increase during the years in the south eastern part of the pond, probably linked to the changed light condition due to the cutting of the trees along one shore of the pond. In 2007, approximately 35% of the pond was covered by *Typha* sp. and *Phragmites* sp. Four fish species, *Pseudorasbora parva*, *Carassus auratus*, *Cyprinus carpio* and *Leucaspius delineatus* were constantly present during the 11 years. The temporary ponds are situated in the forest to a distance from 800 to 1500 m. Their number varies greatly with the precipitation (Hartel *et al*., 2007b), the maximum number being generally recorded in spring. The average pond area is 22 m^2 (range 1-250), the depth varies between 3 to 100 cm (Hartel and Nemes, 2006).

The terrestrial area surrounding the permanent pond is represented by arable lands, a railway, the Târnava River, and a grassland patch between the pond and the forest (Hartel, 2004) (Fig. 1). We created a database regarding the land use types in this area using maps, aerial and other photographies. The landscape composition (in this case the land use types and their amount in the landscape) and configuration (i.e. the spatial arrangement of these) was not changed significantly during the 11 years.

The studies on the permanent pond were started in 1997 whereas in the temporary ponds in 2002. The methodology used for estimating "size of the populations" was presented in detail in Hartel (2004; 2005). The fieldwork started in the middle of February and lasted until the first part of June. Two aspects of the beginning of the breeding season were considered in *R. temporaria* and *B. bufo*: (i) the start of male activity (first day), meaning the first chorus in *R. temporaria* and the first adult males found in the water in *B. bufo* and (ii) the first day when deposited eggs were found. Since it is assumed that females lay maximum one egg mass every year, and the egg masses are easy to count, we used the number of egg masses as indicators of population size in the case of *R. temporaria*. In the case of the Common Toad (*Bufo bufo*) we counted the active individuals in the water until about 1 m from the water shore on land. This method provided ecological meaningful informations about the pond and landscape characteristics influencing the common toads in the Târnava Mare basin (see Hartel et al. 2007b and the references cited therein for the use of "head count" methods"). The population

sizes of Common Spadefoot Toad (*Pelobates fuscus*), and Tree Frog *Hyla arborea* were estimated using the number of calling males.

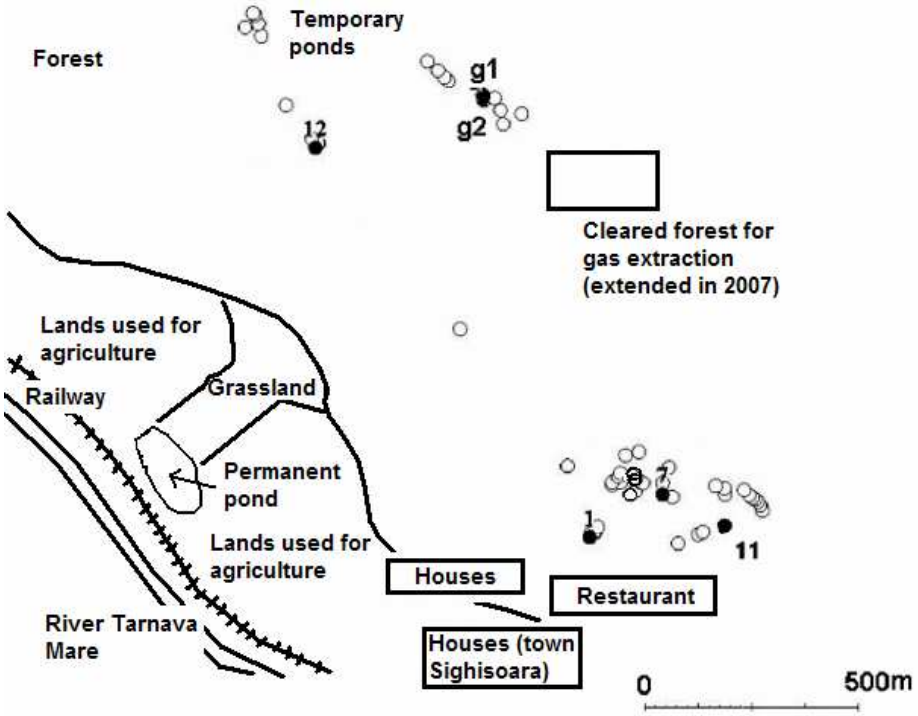

Fig. 1. The study area, the distribution of the ponds and the land use patterns. Black points represent the most stable temporary ponds that were used most frequently. Temporary ponds „g1" and „g2" were used by *Hyla arborea*

We used a number of climatic variables in this study: These variables were: (i) total amount of precipitation (l/mm) in January, and (ii) February in the years when egg masses were counted, (iii) total amount of precipitation during the active season of frogs (April to September) in the year previous to which eggs were counted, (iv) the mean air temperature in January and (v) the mean air temperature in February. These data were measured at the water station from Albeşti, at a distance of about 5 km from the population studied.

Besides the climatic variables (see above), the variables „year" and the number of egg masses recorded for *R. dalmatina* (in the case of *R. temporaria*) (Hartel *submitted*) were used as independent variables in a multipple regression analysis (forward stepwise). The dependent variables in the multipple regression were: the population size estimations (see above), the number of days in the year (using a Julian calendar whereby 1 January is day 1) when the first male called (*R.*

temporaria) or was seen (*B. bufo*) and the Julian date when first egg mass was recorded (both species).

The number of egg masses deposited by *R. temporaria* in the permanent pond and the temporary ponds from the forest was compared using the nonparametric Mann - Whitney U test. The rate of change of population size was estimated using the ΔN method. According to this formula, changes in population sizes between years are related to each other by $\Delta N = \log(N+1)_t - \log(N+1)_{t-1}$, where N is the population size at time t (Houlahan *et al.* 2000).

Results and Discussions

The start of the breeding period. There was no significant difference between the start of the vocalization period in the males and the first egg mass deposited (mean: 71.50 days, SD = 11.84) and the first egg mass (74.22, 10.96) in *R. temporaria* (t = -0.56, df = 20, P = 0.58). In the case of *B. bufo* the differences were significant (males: 75.88, SD = 13.25; females: 88.44, SD = 8.62; t = -2.51, df = 18, P = 0.02). Although not significant in statistical terms (maybe because of low power of the test), the 2.72 day difference between the start of calling and the first egg mass deposited in *R. temporaria* may be significant from a biological point of view. As the number of individuals in the permanent pond is very small (see below) we assume that the vocalization in this case may be important in female attraction. Wells (1977) noted that the male mate locating strategies of explosive breeders, such as *B. bufo* may be dependent on the density. Calling at low density in this species may be advantageous but as the density increases (as more individuals migrate in short time) the calling activity is expected to cease. This was also observed in the population studied by us. In *R. temporaria* however, males in large / dense populations are expected to be more active in the breeding season than in small / less dense populations (Elmberg and Lundberg, 1991). *Bufo bufo* start breeding significantly later than *R. temporaria* (t test, $P < 0.05$). *R. temporaria* always finished breeding in 5-6 days, thus, the overlap between the reproduction periods of two species was minimal. *Bufo bufo* reproduced until the second part of April, active males being found even the first part of May (6^{th} of May). High degree of interspecific spawning was found in the case of *R. temporaria* and *B. bufo* in a pond from England (Reading, 1984). Interspecific pairing between the two species was never observed in these two species due to the short overlap of the breeding seasons. However, the interspecific pairing between the *B. bufo* males and females of *R. ridibunda* were frequently observed. This may be because *R. ridibunda* appears in the pond toward the end of the breeding season of *B. bufo* when the operational sex ratio is strongly skewed toward the males. Size assortative pairing was not observed in *B. bufo* (Hartel and Demeter, 2005) nor in *R. temporaria* (Hartel *unpublished*).

The air temperature in February was the most important variable affecting the start of the reproduction in both species: there was a negative relationship

between the beginning of the reproduction and the air temperature in February (Table 1 and 2), in *B. bufo* the precipitation in January being important determinant of the start of males activity (Table 2). These results confirm the previous findings on the role of the climatic conditions in the initiation of reproduction of temperate explosive-breeding amphibians (Duellmann and Trueb, 1986; Sofianidou and Kyriakopoulou-Sklavounou, 1986; Reading, 1998), including the population of *R. damatina* (Hartel 2005, Hartel *unpublished*). We assume that any long term increase in the air temperature in spring may be reflected by earlier dates at which breeding begins for the populations of these two species. Moreover, the identified climatic variables explains overall a small variation in the begining of thereproduction period of these two species (R^2 is small, the largest value being for the beginning of the males activity in *B. bufo*). This means that other, unrecorded factors may be also important in the start of reproduction of these two species.

Population sizes fluctuations. *Bufo bufo*, and *P. fuscus* used only the permanent pond for reproduction, whereas *H. arborea* occurred in two sunny temporary ponds (Fig. 1), with longer duration. The temporary ponds from the forest are small, shadowed (thus cold and unproductive relatively to the temporary ponds from the open areas) and have short hydroperiod (Hartel *unpublished*), these conditions not being preferred by these two species (Laurila, 2000; Pellet and Hoehn, 2004; Nyström *et al*., 2007).

The average size of the populations of the four species in the permanent pond was: 27.27 (SD = 15.10) egg masses in *R. temporaria*, 1372.80 (SD = 475.69) individuals in *B. bufo*, 46.63 (SD = 23.17) calling males in *H. arborea* and 7.18 (SD = 5.75) calling males in *P. fuscus*. The fluctuation of the population sizes are presented in Fig. 2, 3 and 4. The ΔN is negative in *R. temporaria* (-0.07) and *H. arborea* (-0.07) whereas it is positive in *P. fuscus* (0.09) and *B. bufo* (0.01). The number of the calling males in *H. arborea* and the number of egg masses in *R. temporaria* shows negative trends during the 11 year period (Table 1 and 2). The number of males of *H. arborea* show sharp decline from 2004 (Fig. 3). The number of egg masses deposited by *R. temporaria* was significantly positively associated with the amount of precipitations in January, and negatively associated with the number of the *R. dalmatina* egg masses (Table 1).

It was showed that the sensitivity of females toward the climatic conditions has increased toward the end of the winter dormancy period due to the depletion of energy reserves (Reading, 2007). In dry springs a certain proportion of females may fail to reproduce or mortality could increase.

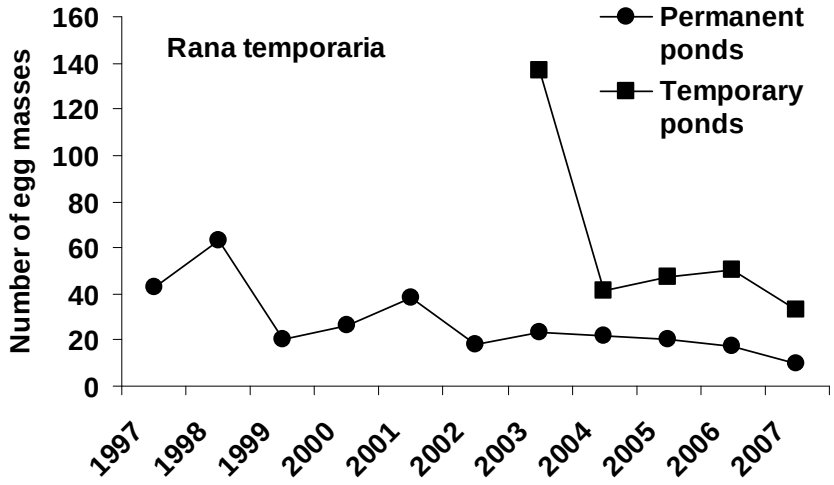

Fig. 2. The fluctuation of the egg mass number in the permanent pond and the temporary ponds in *R. temporaria.* Data from all the temporary ponds are pooled

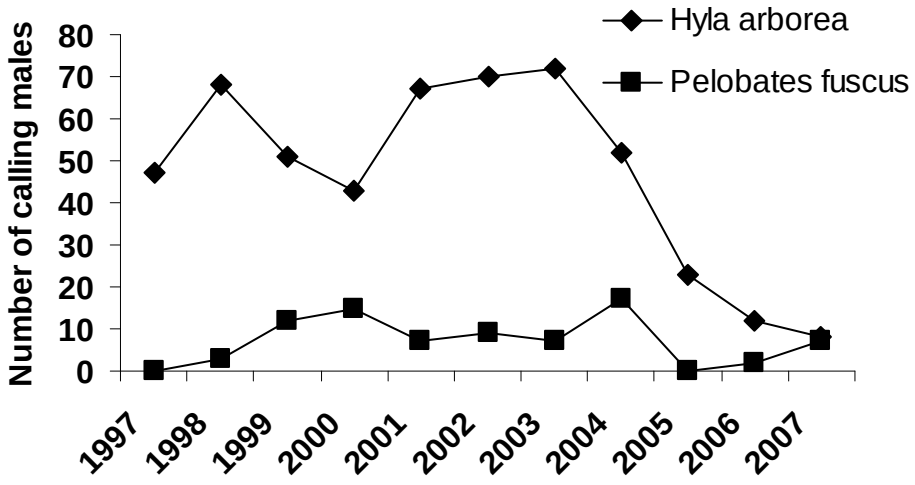

Fig. 3. The fluctuation of the number of calling males in *P. fuscus* and *H. arborea*

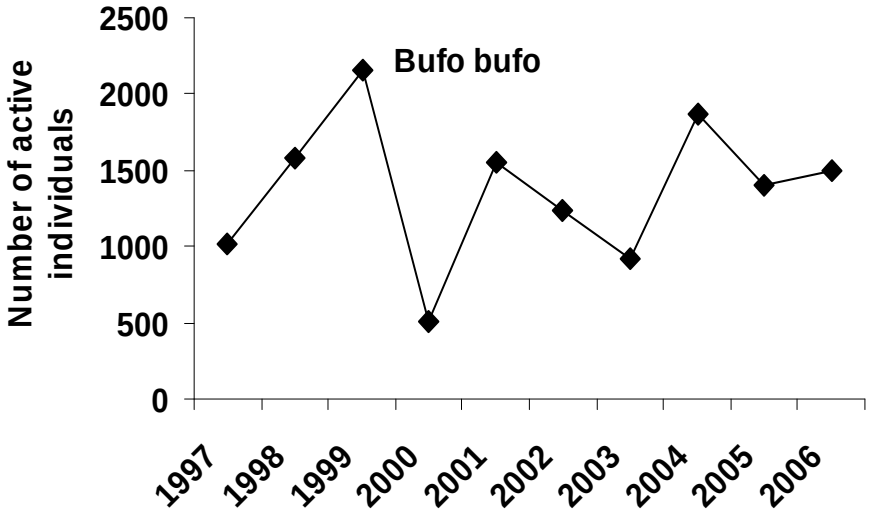

Fig. 4. The fluctuation of the number of active individuals in *B. bufo*

Table 1.

The summary of the multiple regression analysis on the relationship between the climatic variables and the start of reproduction and population size in *Rana temporaria*. The population size of *R. dalmatina* was used as predictor variable for the number of egg masses**.**

Variable	***β* (±SE)**	***t***	***p***	***R²***
Beginning of calling activity				
Air temperature in February	-0.69 (0.23)	-2.93	0.01	0.48
Multiple regression $F_{(1,9)} = 8.60$, $p < 0.01$				
First egg mass				
Air temperature in February	-0.64 (0.25)	-2.51	0.03	0.41
Multiple regression $F_{(1,9)} = 6.33$, $p < 0.03$				
Number of egg masses				
Year	-0.94 (0.15)	-6.14	<0.001	0.85
Population size of *R. dalmatina*	-0.51 (0.15)	-3.34	0.001	
Precipitations in Ianuary	0.43 (0.25)	2.77	0.02	
Multiple regression $F_{(1,10)} = 13.77$, $p < 0.002$				

Table 2.

The summary of the multiple regression analysis on the relationship between the climatic variables and the start of reproduction and population size in *Bufo bufo*

Variable	***β* (±SE)**	***t***	***p***	***R²***
Beginning of males activity				
Air temperature in February	-1.05 (0.24)	-4.31	0.003	0.72
Precipitation January	-0.53 (0.24)	-2.16	0.06	
Multiple regression $F_{(2,8)} = 9.44$, $p < 0.01$				
First egg mass				
Air temperature in February	-0.71 (0.25)	-2.82	0.02	0.59
Precipitation in previous year	0,57 (0.25)	2.28	0.06	
Multiple regression $F_{(1,9)} = 4.27$, $p < 0.05$				
Number of active individuals	No significant effect of climatic variables nor year was found			

Table 3.

The summary of the multiple regression analysis on the relationship between the climatic variables, and the population size in *Pelobates fuscus and Hyla arborea*

Variable	***β* (±SE)**	***t***	***p***	***R²***
Pelobates fuscus				
Temperature January	-0.58 (0.26)	-2.18	0.05	0.34
Multiple regression $F_{(1,9)} = 4.77$, $p < 0.05$				
Hyla arborea				
Year	-0.75 (0.22)	-3.34	0.01	0.67
Precipitation in January	0,70 (0.28)	2.44	0.04	
Multiple regression $F_{(2,8)} = 4.74$, $p < 0.04$				

The number of calling males of *P. fuscus* was negatively associated with the temperature in January whereas in the case of *H. arborea* there was a positive relationship between the number of calling males and the amount of precipitation in January (Table 3). It was suggested that calling in anurans may be energetically demanding behavior the calling intensity of the males being strictly dependent on the energy reserves remained after hibernation (Elmberg and Lundberg, 1991). Thus, what we registered may be the variation of the calling males and not those that survived from year to year. Even in this condition calling may be an indicator of the male's fitness, influencing the probability for finding a mate (Friedl and Klump, 2005). We mention that the recorded climatic variables explained only a small variance of the number of males in *P. fuscus* (34%).

The reasons for the decline of *R. temporaria* and *H. arborea* (in the last species the decline being sharp in the last years) in the permanent pond are not yet known. Both species are susceptible to predation by fish (*Carassus auratus*,

Pseudorasbora parva) (Meyer *et al*., 1998, Teplicky, 2003; Hartel *et al*., 2007c for local study). We have not recorded the changes of the density of these fish species during the years, but it is possible that changes in this variable affected the two species. In the case of *R. temporaria*, the population size was negatively associated with the population size of *R. dalmatina*. *Rana dalmatina* is represented by a large and stable population in this area, and is not negatively associated with predatory fish. Interspecific competition in larval stages may be a cause of the negative correlation between the population sizes of the two species (Riis, 1988). The observations made in the Târnava Mare basin (Hartel *unpublished*) suggest that *R. dalmatina* is more represented in the permanent ponds than *R. temporaria* whereas *R. temporaria* is more efficient in temporary ponds than *R. dalmatina* (Hartel, *unpublished*).

The spatial distribution of the pond use and the reproductive success of Rana temporaria. The average number of the egg masses of *R. temporaria* in the temporary ponds for the five years was 61.60 (SD = 42.64). The median value of the *R. temporaria* egg masses deposited in the temporary ponds were significantly larger (47) than that of those deposited in the permanent pond (22) (Mann – Whitney U test, Z = -2.32, P = 0.02). The reproductive success was more constant in the temporary ponds than in the permanent one. In the permanent pond, reproductive success was recorded only in four years (1997, 1998, 2001 and 2005), these years having larger amount of precipitation in early summer than the other years). In the case of the temporary pond system, the number of the ponds where eggs were deposited varied yearly but metamorphosis occurred in all years (Fig. 5).

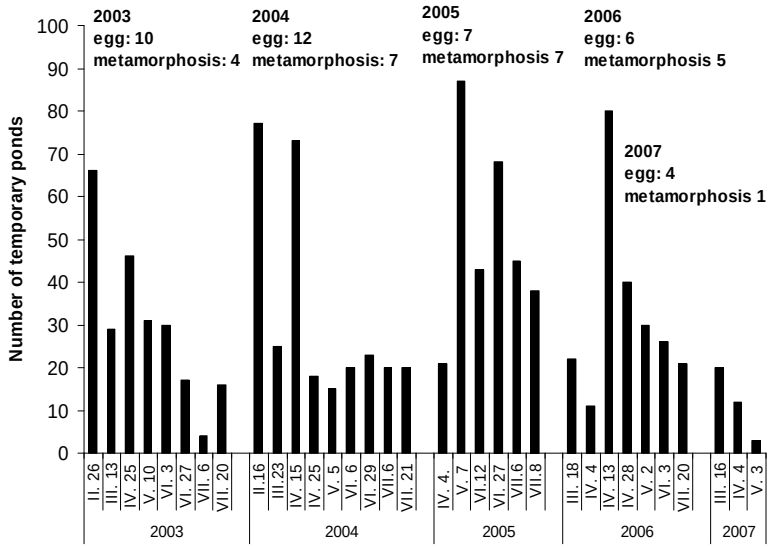

Fig. 5. The fluctuation of the number of temporary ponds after the snow melt until the first part of July (the end of metamorphosis in the majority of *R. temporaria* larvae)

The reproductive success observed in the permanent pond when the water level was increased suggest that the shallow parts of the pond are important for the larval growth and development, most probably due to the temperature regimes, increased productivity and safe against predatory fish. An other temporary pond breeder, *B. variegata* also used this pond for reproduction when the precipitation increased the water level (1998 and 2005) (Hartel, 2004; Hartel *unpublished results*). These results show that the temporary ponds from the forest are more important breeding habitats for *R. temporaria* (probably acting as sources, *sensu* Pulliam 1988) than the permanent pond (that is sink in some years, Pulliam 1988). Temporary ponds are fish free but pose the risk of reproductive failure due to drying. *Rana temporaria* is physiologically adapted to temporary ponds due to its ability to efficiently exploit the resources in temporary ponds and its high phenotypic plasticity (Hartel et al. 2005 and the references cited there).

The number of the temporary ponds with metamorphosis was positively related to the amount of precipitation in May – July (Spearman $r = 0.87$, $P = 0.05$). No relationship was found between the total amount of precipitation in spring (January-April) and the number of ponds used for reproduction. These results show the importance of the precipitations that fill the ponds toward the end of the larval period. At this stage, the larval crowding is high, the nutrient necessity is increased (but the resources are depleted) the waste elements are accumulated and the oxygen concentration is also dropped. According to the model of Wilbur and Collins (1973), there is a threshold larval body size/developmental stage that must be attained to be capable for initiating and completing metamorphosis, in response to environmental deterioration. Refilling the ponds toward the end of the larval stage may contribute to the attainance of the critical development stage at which the metamorphosis can occur. An experimental study (Hartel and Nemes, 2007 in press) indicated that under low energy intake, common frog tadpoles from this population fail to adapt to changing environmental conditions decreasing not only the chance of survival until metamorphosis but the post metamorphic fitness as well. The highest mortality was observed between the larvae having 34 - 38 Gossner stage (Gossner, 1960) (Hartel and Nemes, *in press*).

In conclusion, the climatic variables in spring are good predictors for the number of reproducing adults in *R. temporaria*, *P. fuscus* and *H. arborea*. *Rana temporaria* and *H. arborea* shows negative trends. It seems that the aquatic habitats from this landscape do not provide good habitats for *P. fuscus* and *H. arborea* the reasons not being known. This and the previous studies (Hartel, 2005; Hartel *et al.*, 2007a) show that maintaining a large variety of aquatic habitats at landscape scale is crucial for amphibian communities. Some species are more represented in the temporary ponds (i.e. *B. variegata* [Hartel *et al.*, 2007a], *R. temporaria* [this study]) whereas other species use the permanent pond (i.e. *B. bufo*). As it was suggested before (Hartel, 2004; Hartel and Demeter, 2005) the maintenance of the

green corridor between the permanent pond and the forest (the grassland in the Fig. 1) is crucial for this amphibian community.

We strongly encourage studies that aim to find how natural populations "behave" in different landscape types in Romania. Further studies will elucidate if the protected status of this landscape will really contribute to the conservation of this amphibian community.

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THE BREEDING AVIFAUNA OF THE "BREITE PLATEAU" NATURAL RESERVE AND THE SURROUNDING FOREST

COSMIN IOAN MOGA[1,3], KINGA ÖLLERER[2] AND TIBOR HARTEL[3]

SUMMARY. This paper presents the breeding avifauna of the Breite plateau in comparison with the breeding avifauna of the surrounding forest, accounting for alpha, beta and gamma diversity. The bird species are considered from the perspective of their feeding habits, nest site selection and protective status. 57 breeding bird species were identified, from which 32 species on the plateau, 46 species in the surrounding forest and 21 species were found breeding in both habitats. Due to its small area and elongated shape, the plateau has a large ecotone and the surrounding forest has a great influence on its avifauna. 21 of the bird species that were found in both habitats are forest species. Only 11 species are breeding exclusively on the plateau and all are open habitat species. 25 species nest exclusively in the forest, indicating again the dominance of forest species over the total species number. The bird species from the two studied habitats belong to five trophic categories, the insectivores being dominant in both cases. The several century-old oaks from the plateau represent important feeding habitats for insectivore birds, especially for the bark-feeders. The cavity nesters are the best represented among the species breeding on the plateau. The open nests builders are best represented among the forest-nesting birds. Those that nest on soil are less represented in both habitats. Out of the 32 species that are breeding on the plateau, six are protected according to the Birds Directive (79/409 EEC), Annex I, emphasizing the conservation importance of the Breite reserve.

Keywords: breeding avifauna, woodpasture, forest.

Introduction.

Forests, woodlands and woodpastures are globally threatened habitats especially because of forestry, lack of regeneration, continuous expansion of agricultural fields and overgrazing (Wilson *et al.*, 1991; Zack *et al.*, 2002; Goldberg *et al.*, 2007). The majority of these habitats still maintained in Europe have small dimensions, up to 50 ha (Opdam *et al.*, 1984; Bellamy *et al.*, 1996; Hansson, 1997; Goldberg *et al.*, 2007).

"Old" habitats of this kind, with secular trees and an undisturbed vegetation cover, are more structurally heterogeneous and more valuable from the perspective of harbored diversity of organisms. The structural heterogeneity of these habitats and their higher biodiversity is a consequence of a small-intensity anthropic impact and of a moderated disturbance regime that characterized the

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interactions between nature and humans in the past (Peterken, 1983). The high structural heterogeneity of these woodpastures is also correlated with their size, the species-area relationship having a major influence on the diversity of occurring bird species (Bellamy at al., 1996; Hansson, 1997). Even trees with a dispersed distribution and coverage of six to ten percent are important for the species diversity, being key elements both at the local and at the landscape level (Manning *et al.*, 2006).

In this paper we present: (1) the breeding avifauna of the Breite plateau, in comparison with the breeding avifauna of the surrounding forest, counting for alpha, beta and gamma species diversity (Whittaker, 1972), (2) the trophic analysis of the avifauna, (3) nest site selection of species, (4) data regarding the protective status of the avifauna from the Breite plateau.

Study area

The study area is situated nearby the town of Sighişoara, at a distance of around 2 km and at an altitude of 504 m in the northern part and 530 m in the southern part of the plateau. Its geographical coordinates are 46°13'05 N and 24°45'18 E in the most northern part and 46°11'03 N and 24°45'14 E in the most southern part.

The Breite reserve (Fig. 1) is a woodpasture, where most of the trees are several century-old oaks (*Quercus robur* and *Q. petraea*, hybrids in the majority) that cover around 7 % of the total area (133 ha) of the plateau. On a few smaller patches the tree cover is up to 65 %, these areas having a woodland character. The habitat has anthropic origins, being created in the Middle Ages by the inhabiting Saxon community in order to increase the acorn production. The thinning of the original forest has favored the penetration of mesophilous meadows with *Alopecurus pratensis* and *Sanguisorga officinalis*, while the relatively recent (starting from the mid '80s) desiccation has led to the expansion of *Dechampsia caespitosa.* Shrubs are more scarcely present, most of them being *Salix spp, Rosa canina* and *Crataegus monogina* individuals.

The plateau has an elongated shape in the north-south direction and its structure is influenced by the surrounding mixed deciduous forest (*Quercus petraea* and *Carpinus betulus* with *Fagus sylvatica* individuals). The hornbeam (*Carpinus betulus*) invasion, from the surrounding mature forest, is frequent on the plateau, covering around 10 % of it.

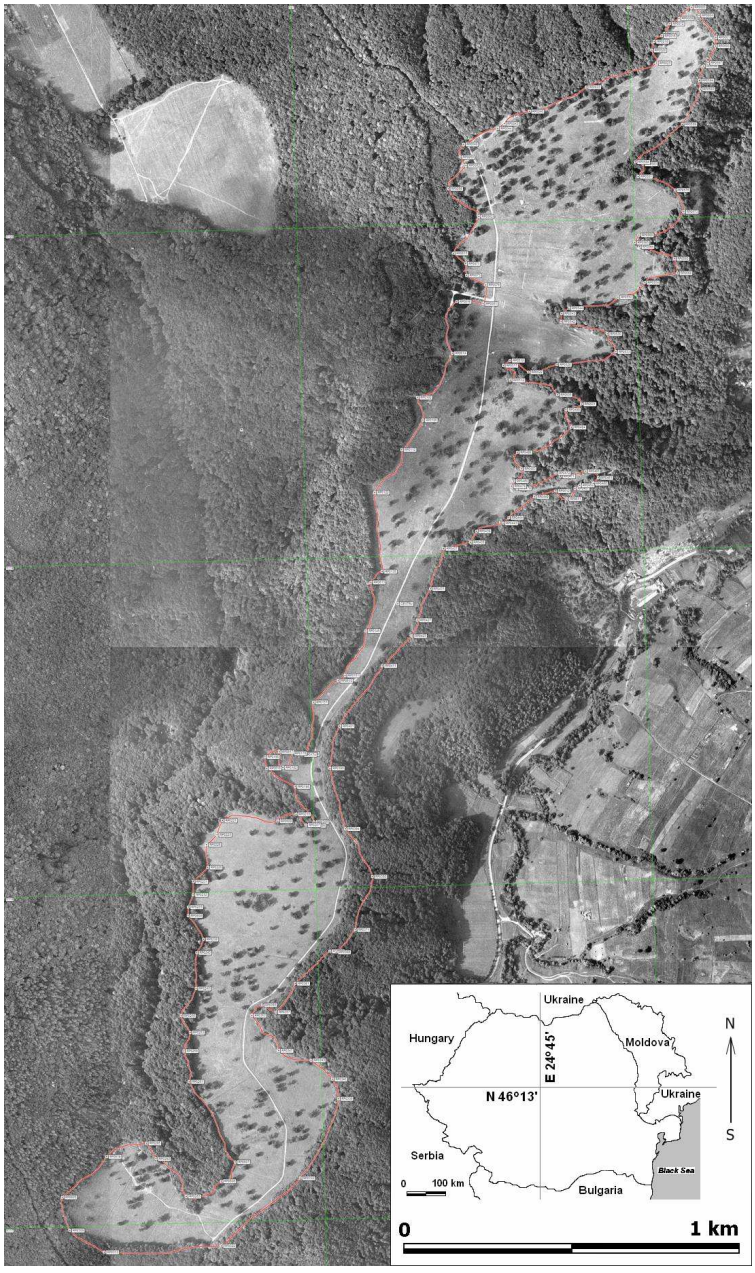

Fig. 1. Localization and aerial photo of the Breite plateau

Methods

The observations on the avifauna were effectuated in the 2003-2007 period. The line transect was used in combination with point count observations (Bibby 2000), depending on the accessibility in the field. The observations were done in the morning, immediately after sunrise, until 10.30 a.m. The species were identified visually and based on the singing of males. 37 observations were done both on the plateau (woodpasture habitat) and in the surrounding forest.

The inclusion of birds in different trophic categories was based on the obvious adaptations of the bill and legs, considering the basic trophic regime of the species and not the particular feeding situation during the reproduction period, when most species feed upon resources of animal origin, especially insects.

When analyzing the nest site selection of the different species, we considered three situations: nests built on the ground; nests built on herbaceous vegetation, shrubs, trees, mentioned in the text as open nests; and cavity nests, that were built in hollows, scooped by birds (woodpeckers), or built under the bark of trees or in other cavities (tits).

Regarding the protective status, we noted the breeding bird species that are protected according to the Directive 79/409/EEC (Birds Directive), Annex I.

The species breeding on the plateau and in the forest were coded using binary variables (0 for the absence of breeding species, 1 for presence). The two habitats were compared using Cochran Q Test.

Results and Discussions

The avifauna of the Breite plateau in comparison with avifauna of the surrounding forest. In total, on the plateau and in the surrounding forest, we recorded 57 breeding bird species (gamma diversity) (Table 1, Fig. 2,). Out of these, 21 species (36.84 %) were found nesting both on the plateau and in the surrounding forest. The forest has more breeding species (alpha diversity for the forest = 46) in comparison with the plateau (alpha diversity for the plateau = 32) (Fig. 2,), the differences being significant ($Q = 5.44$, $df = 1$, $p < 0.01$).

Out of the total 57, those 32 species that are nesting on the plateau represent 56.14 %. In this percentage both the exclusive breeders and the species that are breeding in both habitats are included. Other studies revealed similar results. Hansson (1997), recorded 34 breeding bird species in several oak-hazel woodland habitats from Sweden, with sizes of 0,2-12 ha. Brawn (2006), in a oak savanna habitat from Illinois (North America), with canopy closure between 10 % and 70 % (similar to our study site), recorded 31 bird species.

Out of the total 57, 11 species (19.29 %) are nesting exclusively on the plateau. These are open habitat species (see Table 1) and their small number can be explained by the relatively small area of the plateau (133 ha) and especially by its elongated shape, with a small area/edge ratio, between the small interior and the large ecotone. For this reason, the surrounding forest has a major influence on the avifauna of the

plateau, the species found in both habitats (21 species, see below) being all forest species, especially forest edge species. Out of the 32 species that are nesting on the plateau, those 11 exclusive breeders represent 34.37 %, and those that are breeding in both habitats represent 65.62 %, being an evidence of the dominance of forest species in the composition of the plateau's avifauna.

Table 1.

The identified bird species, their trophic regime and nesting site selection

Species	**Breeding exclusively on the plateau**	**Breeding exclusively in the surrounding forest**	**Breeding in both habitats**	**Trophic regime**	**Nesting site**
Ciconia nigra	-	*	-	Zooph. Pol	Op
Aquila pomarina	-	*	-	R	Op
Buteo buteo	-	*	-	R	Op
Pernis apivorus	-	*	-	R	Op
Accipiter gentilis	-	*	-	R	Op
Accipiter nisus	-	*	-	R	Op
Columba oenas	-	*	-	Omn	Cav
Columba palumbus	-	*	-	Omn	Op
Cuculus canorus	-	*	-	In	Op
Bubo bubo	-	*	-	R	Gr
Strix uralensis	-	*	-	R	Cav
Asio otus	-	*	-	R	Op
Strix aluco	-	*	-	R	Cav
Upupa epops	*	-	-	In	Cav
Picus viridis	-	-	*	In	Cav
Picus canus[1]	-	-	*	In	Cav
Dendrocopos major	-	-	*	In	Cav
Dendrocopos syriacus[1]	-	-	*	In	Cav
Dendrocopos medius[1]	-	-	*	In	Cav
Dendrocopos minor	-	-	*	In	Cav
Dendrocopos leucotos	-	*	-	In	Cav
Dryocopus martius[1]	-	-	*	In	Cav
Jinx torquilla	-	-	*	In	Cav
Anthus trivialis	-	-	*	In	Gr
Motacilla alba	*	-	-	In	Gr
Lanius collurio[1]	*	-	-	Zooph. pol	Op
Lanius excubitor	*	-	-	Zooph. pol	Op

Table 1 (continued)

Oriolus oriolus	-	-	*	In	Op
Sturnus vulgaris	-	-	*	Omn	Cav
Garrulus glandarius	-	*	-	Omn	Op
Corvus corax	-	*	-	Omn	Op
Troglodytes troglodytes	-	-	*	In	Op
Sylvia borin	-	-	*	In	Op
Sylvia atricapilla	-	*	-	In	Op
Sylvia curruca	*	-	-	In	Op
Phylloscopus trochilus	-	*	-	In	Op
Phylloscopus collybita	-	-	*	In	Op
Phylloscopus sibilatrix	-	*	-	In	Op
Ficedula albicollis[1]	-	-	*	In	Cav
Ficedula parva	-	*	-	In	Cav
Saxicola rubetra	*	-	-	In	Op
Saxicola torquata	*	-	-	In	Op
Phoenicurus phoenicurus	-	*	-	In	Cav
Erithacus rubecula	-	*	-	In	Op
Turdus merula	-	*	-	Omn	Op
Turdus philomelos	-	*	-	Omn	Op
Parus palustris	-	-	*	Omn	Cav
Parus coeruleus	-	-	*	Omn	Cav
Parus major	-	-	*	Omn	Cav
Aegithalos caudatus caudatus	*	-	-	Omn	Op
Aegithalos caudatus europaeus	*	-	-	Omn	Op
Sitta europea	-	-	*	In	Cav
Certhia familiaris	-	*	-	In	Cav
Passer montanus	*	-	-	Omn	Op
Fringilla coelebs	-	-	*	Veg	Op
Coccothraustes coccothraustes	-	-	*	Veg	Op
Carduelis chloris	*	-	-	Veg	Op
Total 57 species	**11**	**25**	**21**		

Abbreviations: R = raptor, Zooph. pol = zoophagous polyphagous, In = insectivores, Omn = omnivores, Veg = vegetarians; Gr = nest on the ground, Op = open nests, Cav = cavity nests. Species marked with [1] are protected according to Birds Directive, Annex I.

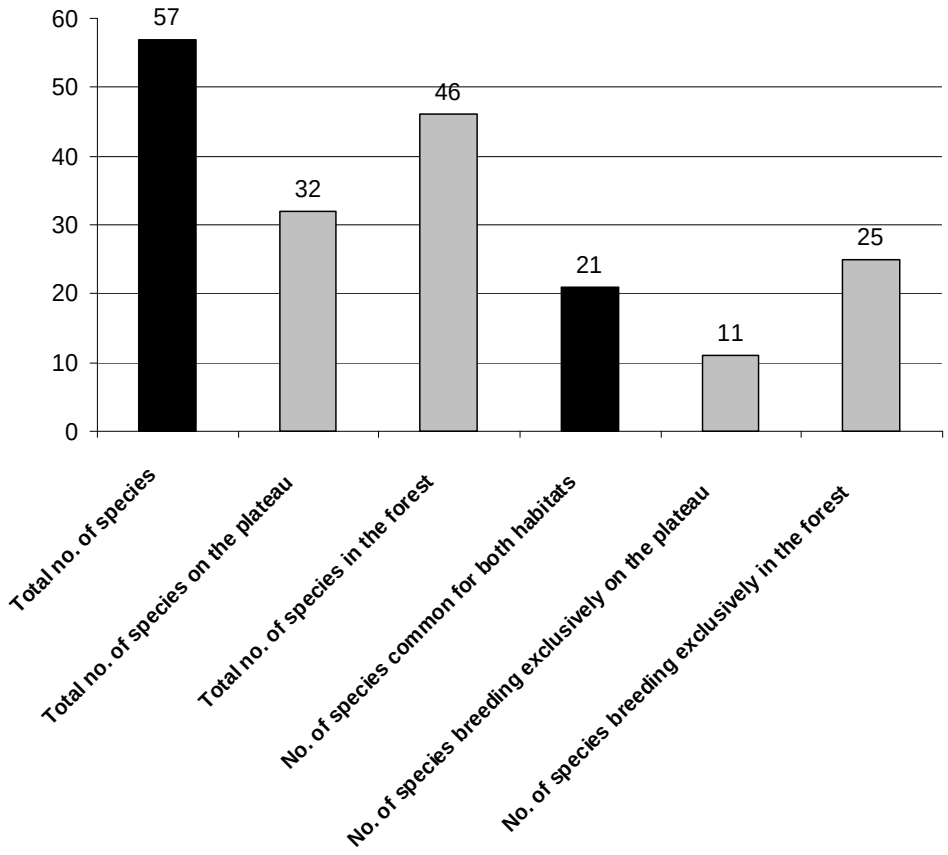

Fig. 2. The total species number and their number in the studied habitats

46 out of the 57 species that are nesting in the surrounding forest represent 80.70 %. In this percentage both the species that are nesting exclusively in the forest (see below) and the species that are nesting in both habitats (21 species) are included. At the landscape level, the forest species are more abundant in number because of the large area of the surrounding forest (in comparison with the studied woodpasture) and its heterogeneity. Laiolo (2002) showed that natural, mature forests are habitats that contribute to the maintenance of a high biodiversity level. Out of the total 57, 25 (43.85 %) species are nesting exclusively in the forest; this is a much higher percentage than that of the species that are exclusive breeders on the plateau, which, again, emphasizes the dominance of forest species in the total number of species. From the 46 species that are nesting in the forest, those 25 species that are nesting exclusively in this habitat represent 54.34 % and the 21 species that are breeding in both habitats account for 45.65 %.

The beta species diversity for the two habitats is 36 (11 species that are exclusive breeders for the plateau + 25 species breeding only in the forest).

Trophic analysis of the avifauna: The bird species from the studied habitats belong to five trophic categories, the insectivores being dominants in all cases (Table 2). The insectivores are the most abundant (71.42%) among the 21 species that are breeding in both habitats. Among the 32 species that are breeding on the plateau, the insectivores are again well represented (62.5%), while among the 46 species that breed in total in the forest, the insectivores account only for 52.17%. Among the 11 species nesting exclusively on the plateau the insectivores represent 45.45%, while among the 25 exclusive forest nesters they represent only 36%. In a study on the avifauna of several habitats on the Mediaş table-land (1000 Km^2), that includes also the Breite plateau, only 16 insectivore bird species were found in the open habitats (Moga 2005), less than in the present study, although the area studied then was much larger. These results are explained by the presence on the Breite plateau of birds that belong to the surrounding forest habitats (see Table 1). Besides the nesting sites they provide, the several century-old oaks represent a food resource for the birds, especially for the insectivores, being key habitats for the maintenance of the bird species diversity in the woodpasture (Manning *et al.*, 2006). Among the insectivores, the best represented are the bark-feeders (see Table 1). Out of the 10 woodpecker species that are nesting in Romania, 9 species were found nesting on the plateau and in the surrounding forest (see Table 1). Only *Picoides tridactylus* is missing, but this is a mountain spruce forest species. Globally, the insectivores, most of them migratory species, show the largest fluctuations in density and are also the most exposed to extinction risk (Newton, 2004).

Raptors are nesting only in the forest, the area of the plateau being too small and the anthropic disturbance too large (Moga, pers. comm.) in comparison with the nesting necessities of these species. The omnivores are the best represented among those 11 species that are nesting exclusively on the plateau (27.27%). The other trophic categories are more weakly represented in number (see Table 2).

By its setting, being surrounded by mature deciduous forests, the Breite plateau creates spatial heterogeneity at the landscape level, being of interest not only for the bird species nesting on the plateau, but also for the birds with double territory, like the small and medium sized raptors, for which it represents a feeding territory (Moga, 2005). The big raptors, ex. *Aquila pomarina*, do not use the habitat for feeding, because of its small area. This species can be seen here only accidentally; although it is nesting in the forests that surround the plateau, it prefers the larger, opened areas of the Şaeş Valley, at about 2 km distance from the plateau. At the landscape scale, the support capacity of an area for raptors is given by the existence of two types of resources: feeding and nesting sites. The decrease of one of these two resources results in a decrease of the raptor population (Newton 2002).

Table 2.

Trophic analysis of the breeding avifauna of the two habitats

	Total no. of species breeding on the plateau	No. of species breeding exclusively on the plateau	Total no. of species breeding in the forest	No. of species breeding exclusively in the forest	No. of species breeding in both habitats
Total no. of species	32	11	46	25	21
Raptors	0	0	9 (19.56%)	9 (36%)	0
Zoophagous polyphagous	2 (6.25%)	2 (18.18%)	1 (2.17%)	1 (4%)	0
Insectivores	20 (62.5%)	5 (45.45%)	24 (52.17%)	9 (36%)	15 (71.42%)
Omnivores	7 (21.87%)	3 (27.27%)	10 (21.73%)	6 (24%)	4 (19.04%)
Vegetarians	3 (9.37)	1 (4.34%)	2 (4.34%)	0	2 (9.52%)

Table 3.

Analysis of bird species breeding in the two habitats according to the nest site selection

	Total no. of species breeding on the plateau	No. of species breeding exclusively on the plateau	Total no. of species breeding in the forest	No. of species breeding exclusively in the forest	No. of species breeding in both habitats
Total no. of species	32	11	46	25	21
Nest on the ground	2 (6.25%)	1 (9.09%)	2 (4.34%)	1 (4%)	1 (4.76%)
Open nests	15 (46.87%)	9 (81.81%)	23 (50%)	17 (68%)	6 (28.57%)
Cavity nests	15 (46.87%)	1 (9.09%)	21 (45.65%)	7 (28%)	14 (66.66%)

Breeding site selection: Among the 21 species that are nesting in both habitats, the cavity nesters represent 66.66% (Table 3). Also, these are well represented (46.87%) among the 32 species that are nesting on the plateau. The structural heterogeneity of the surrounding mature forest, together with the several century-old hollowed oaks from the plateau, offer various nesting sites for those in search of cavities. There is a positive correlation between the species diversity, species richness, the number of hollow nesters, the number of species from the forest interiors and the forest age (Laiolo 2002).

The species that build open nests are more abundant (50%) in the forest habitat. Those that are nesting on soil are weakly represented in both habitats (Table 3).

The protective status of the avifauna. Out of the 32 species that are breeding on the plateau, six are protected according to the Birds Directive (79/409

EEC), Annex I (see Table 1), representing 3.42 % of the total number of species protected according to this directive at European level. Considering the relatively small area of the plateau, we believe that the species that are categorized as important, based on their protective status, are well represented among the overall avifauna.

Conclusions

Because of the small size, elongated shape and large ecotone of the area, the composition of the plateau's avifauna is strongly influenced by the surrounding forest, only 11 species (19.29 % out of the total 57 species) being exclusive breeders for the plateau, all of them open habitat species. The 21 species that were found breeding in both habitats represent 36.84 % of the total 57 species; all of them forest habitat species. 25 species (43.85 %) are exclusive breeders for the forest.

The bird species from the two studied habitats belong to five trophic categories, the insectivores being dominant in both cases. The several century-old oaks represent important feeding habitats for the insectivore birds, especially for the bark-feeders.

Cavity-nesters are the best represented as percentage (46.87%) among the 32 species that are nesting on the plateau. The species that build open nests are the best represented (50%) among the forest nesting species. Those that are nesting on the ground are weakly represented in both habitats.

Out of the 32 bird species breeding on the plateau, six are included in the Birds Directive (79/409 EEC), Annex I, the plateau holding a relevant number of species of conservation interest.

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===REVIEW===

CAROTENOIDS IN BIRDS' LIFE

CRISTINA BERCIU[1] AND ALIN DAVID[1]

SUMMARY. Carotenoid pigments are biologically active, fat-soluble compounds that are responsible for red, orange and yellow secondary sexual coloration in many animal species. Besides, they act as antioxidants and stimulate various aspects of immune function, detain photoprotective activities, enhance motor performance and cognition, and improve intercellular (gap-junctional) communication. Carotenoid availability may be physiologically limiting, because animals cannot synthesize carotenoids *de novo*, and they must obtain them through diet, but also because there may be trade-offs in the allocation of carotenoids between competing somatic demands such as sexual display and other functions. Unfortunately, all these aspects, and functions, essential for the individuals themselves and their offsprings, too, are insufficient or less known. According to the previous studies, this paper is a review concerning the essential information accumulated over the past years which may catalyze future lines of research on the profile and functions of carotenoids in birds (in different moments of their life and in different structures), and finally, the possibility to use the useful information to the bird's benefit.

Keywords: antioxidant, carotenoids, immunostimulatory, intercellular communication, motor performance, sexual coloration

Introduction

Among the various pigments which are involved in ornamental colors in birds, the carotenoids have a special, major role. Carotenoids are the second most prevalant pigment in the avian integument (melanin being the first). They are an extremely large and diversified group of lipid-soluble hydrocarbons, and are responsable for red, orange and yellow secondary sexual coloration of the plumage, and also of the skin, scales, beaks, combs, wattles, and eyes round out the avian integumentary tissues.

Birds cannot synthetize carotenoids *de novo* (because they lack the enzyme to manufacture carotenoids from the precursors of these ones), so they have to obtain the necessary carotenoids through their diet (consuming algae, fungi, plant parts, insects, crustaceans, vertebrates which contain these pigments), while some others are produced through the metabolism of certain carotenoids in their diet (Brush, 1990; Moller *et al.*, 2000). Thus, many of the carotenoids found as plumage, leg and beak colorants are not present in the diet. These metabolic processes are thought to factor prominently into information content, signal function and evolutionary history of carotenoid-based ornamental coloration.

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The mechanisms of the absorbtion from food, circulation and tissue storage of carotenoids are incompletely known, but, so far, it is very well known that the intestinal absorbtion of carotenoids involve some special receptors of the enterocytes at the level of duodenum, jejunum and ileum. Once they are retrieved from food and fractioned with lipids (as micelles) in the intestinal mucosa, carotenoids are packaged into chylomicron fractions and enter the lymphatic system, where they are incorporated into lipoproteins that circulate through the bloodstream (Williams *et al.*, 1998).

Intraspecific differences of the carotenoids in plasm and skin are induced by the geographic and temporally differences concerning the food quality (dietary availability of carotenoids) of populations belonging to a species (Partali *et al.*, 1987; Hill, 1993; Linville and Breitwisch, 1997; Negro and Garrido-Fernandez, 2000).

Sex diferences in carotenoid circulation are not a function of diet, but instead likely come under physiological control, through the action of lipoproteins and such sex-steroid hormones and testosterone, as well as the need for females to shunt carotenoid pigments to egg yolk (Blount *et al.*, 2000; Hill, McGraw, 2006).

Carotenoids accumulate at highly variable concentration in the serum of the wild birds (0,5-75 µg/ml). Thus, in an ecological and evolutionary comparative analysis of 80 species, the best predictors of plasma carotenoid concentration were diet, extend of carotenoid-based plumage, body mass. Species with the highest carotenoid values that fed on predominately plant-based diets, exibited more plumage are that was pigmented with carotenoids and tended to be smaller (Tella, 2004; Hill and McGraw, 2006).

Having absorbed the carotenoids from the intestinal lumen, they will be transported to different tissues and metabolized. Liver is the most important organ involved in the metabolism of these pigments (Brush, 1990), but, also the small intestine, kidneys and lungs. At the level of all these organs, carotenoids are converted to pro-vitamin A (Wiss, 2004). A few data support the idea that birds make their colorful display carotenoids in the liver. But, there are a lot of studies which demonstrated that metabolically derived carotenoids present in the feathers and bare parts are not found in the liver or serum of these birds, they being produced in maturing feather folicles or beak/leg keratinocytes (McGraw, 2004). Very often, birds do not metabolize carotenoids for coloration purposes and, instead, directly deposit dietary and serum carotenoids into feathers and bare parts, because the process of carotenoids' metabolization needs a lot of energy (Hill and McGraw, 2006). In other cases, only one sex (typically the male) is capable of metabolizing carotenoids (Hill and McGraw, 2006).

Carotenoids procured from the diet will be delivered (metabolized or unmetabolized) by blood circulation to different tissues and organs. But, so far, there are just a few studies concerning the differences between the storage preferences of these pigments and species or type of carotenoid.

Storage of the carotenoids is characteristic especially for the feathers, skin, adipose tissue, liver, egg yolk, retina and ovary (ovaries are said to contain about half of the body's carotenoid reserves in female). Testes, semen, heart, kidney, breast/leg

muscles, bile and lung also house measurable amounts of carotenoids. Some carotenoids may cross the blood-brain barrier. So, these pigments can be detected at the level of the nervous tissue, where they are involved in very important processes (protect neurons from oxidative damage induced by free radicals which are produced as a consequence of the fat acids' metabolism, slow the rate of apoptosis of neurons, enhance cellular signaling and transcriptional regulation, improve temporal and spatial memory, enhance motor performance or cognition (Sumien *et al.*, 2004).

So far, there is no evidence that a certain type or types of carotenoid(s) are allocated to one or a few tissues. (Hill and McGraw, 2006). Tissue levels of carotenoids may depend on the amounts of these pigments in diet, and also on total tissue mass. For example the highest concentrations of carotenoids in adipose tissue is correlated with the smallest fat stores (Negro *et al.*, 2001).

Besides, it could be noticed many sex differences in internal-tissue carotenoids (Negro *et al.*, 2001). Many monogam species are characterized by males with a brighter carotenoid-based coloration than do females. There may be other important reasons why individuals and species vary in their ability to endogenously store and retrieve carotenoids from the tissues.

So far, there are many studies which demonstrated the existence of differences between bird species concerning their capacity to accumulate and mobilize tissue carotenoid repositories for self or offspring defense from oxidative damage. The most stable amounts of carotenoids are those in liver. Carotenoids in liver may be a mobilizable, expendable pool of carotenoids for use during infectious challenges, although, usually, they are more stable and required for local cell and tissue maintenance (Koustos *et al.*, 2003b).

Functions of carotenoids. Carotenoid storage (as they are found in food or metabolized) at the level of different tissues is correlated with the numerous functions which they have: create the ornamental coloration in birds, act as antioxidants, stimulate various aspects of immune function, guarantee a photoprotection, stimulate the cellular diferentiation and communication.

As we already mentioned, carotenoids can be incorporated into several different tissue types to **give birds external colour**, which are very important in sexual behavior and selection. Besides, recent researches noted that carotenoid-based feathers reflect a substantial amount of ultraviolet light (Hill and McGraw, 2004). In the beginning, it is thought that carotenoids are responsable to give the external color of feathers, skin, scales, beaks, combs, wattles and eyes round out the avian integumentary, but, later, Olson and Owens (2005) showed that carotenoid pigmentation is typical for bird plumage, but the use of these pigments in bare part pigmentation may have a different functional basis and may be more strongly influenced by genetic and physiological mechanisms, which currently remained relatively understudied.

Carotenoids are coloured pigments because of their conjugated double-bound system, known as the " chromophore", which absorbs particular wavelengths of light and gives colour to the molecules based on the degree of conjugation in the hydrocarbon chain and end-rings. Molecules with more conjugated double bonds absorb more short wavelengths of light and thus are redder in colour.

Some carotenoids (astaxanthin, cantaxanthin, adonirubin, α-doradexanthin) confere a red colour of the tissues or structures, while others, more exactly: xanthophylls (lutein, zeaxanthin and canary xanthophylls) co-ocur in yellow tissues. Involving of carotenoids in appearance of yellow colour is bigger than in red colour (Olson and Owens, 2005). Always, more than a single carotenoid type typically appears in any given integumentary tissue in birds. For example, red feathers and bare parts often house a collection of 4-oxocarotenoids, and the canary xanthophylls co-occur in yellow tissues. There is some exceptions to this rule, of course. Lutein is the lonely colorant of yellow feathers in songbirds (Hill and McGraw, 2006). Carotenoid colors can not only affected by the types, amounts and ratios of carotenoid pigments that occur in the integument, but also by their co-occurrence with other types of pigments or structural mechanisms and by the nature of their physical interactions with tissues (Hill and McGraw, 2006).

The table bellow (Table 1) reflects the bird species in our country for which carotenoids have been identified from colorful feathers and bare parts.

Table 1.

Bird species also found in Romania for which carotenoids have been identified from colorful feathers and bare parts (modified from Hill and McGraw, 2006)

Order/ Family	**Species**	**Trait**	**Major carotenoids**
Anseriformes	Mallard *(Anas platyrhynchos)*	Orange tarsi	1, 2, 4, 12, 15[a]
	Greylag Goose *(Anser anser)*	Orange tarsi	3, 4, 12[b]
Falconiformes	Egyptian Vulture *(Neophron percnopterus)*	Yellow facial skin	1
Galliformes	Domestic Chicken *(Gallus gallus)*	Red comb	1, 4, 12[b]
		Yellow tarsi	1, 2, 3, 4, 12, 15[b]
	Wild Turkey *(Meleagris gallopavo)*	Red comb	3, 4, 12[b]
		Orange-brown tarsi	1, 2, 3, 4[b]
	Grey Partrige *(Perdix perdix)*	Orange-brown tarsi	1, 3, 12, 15
	Ring-necked Pheasant *(Phasianus colchicus)*	Orange-brown tarsi	1, 3, 12, 15
		Red facial comb	12
	Capercaillie (*Tetrao urogallus*)	Red supraocular comb	1, 2, 12, 14

Table 1 (continued)

Order/ Family	Species	Trait	Major carotenoids
Ciconiiformes	White Stork *(Ciconia ciconia)*	Red bill and tarsi	12
Piciformes	Great Spotted Woodpecker (*Dendrocopos major*)	Red plumage	12, 13, 14
	Three-toed Woodpecker (*Picoides tridactylus*)	Yellow plumage	1, 2
	Green Woodpecker (*Picus viridis*)	Yellow plumage	1, 2, 22, 24
		Red plumage	12, 13, 14, 15
Passeriformes/ Corvidae	Golden Oriole (*Oriolus oriolus*)	Yellow plumage	1, 2
Passeriformes/ Bombycillidae	Bohemian Waxwing *(Bombycilla garrulus)*	Yellow tail band	6, 7
Passeriformes/ Muscicapidae	Robin *(Erithacus rubecula)*	Orange plumage	1, 10
Passeriformes/ Sittidae	Wallcreeper *(Tichodroma muraria)*	Red plumage	12
Passeriformes/ Paridae	Great Tit *(Parus major)*	Yellow plumage	1, 2
	Blue Tit *(Parus caeruleus)*	Yellow plumage	1, 2
Passeriformes/ Aegithalidae	Long-tailed Tit (*Aegithalos caudatus*)	Pink plumage	16
Passeriformes/ Regulidae	Goldorest *(Regulus regulus)*	Yellow plumage	1, 2
		Red plumage	12, 13, 14
Passeriformes/ Passeridae	Yellow Wagtail *(Motacilla fiava)*	Yellow plumage	1, 2
Passeriformes/ Fringillidae	Chaffinch *(Fringilla colebs)*	Yallow plumage	1, 2
	Brambling *(Fringilla montifringilla)*	Yellow plumage	1
	Eurasian Bullfinch *(Pyrrhula pyrrhula)*	Red plumage	12, 13, 14, 15, 21
	Red Crossbill *(Loxia curvirostra)*	Yellow plumage (f)	6, 7
		Red plumage (m)	16, 19, 20
	Citril Finch *(Serinus citrinella)*	Yellow plumage	6, 7
	European Serin *(Serinus serinus)*	Yellow plumage	6, 7
	European Greenfinch *(Carduelis chloris)*	Yellow plumage	1, 6, 7
	Common Redpoll *(Carduelis flammea)*	Red plumage	12, 14, 15, 16
	Linnet *(Carduelis cannabina)*	Red plumage	12, 14, 15, 16
	Hoary Redpoll *(Carduelis bornemanni)*	Red plumage	12, 14, 15, 16, 19
Passeriformes/ Emberizidae	Yellowhammer *(Emberiza citrinella)*	Yellow plumage	1, 2
Passeriformes/ Icteridae	Eurasian Blackbird *(Turdus merula)*	Orange-yellow bill	1, 2, 3, 4, 5

Notes: f-female; juv-juvenile;m-male; 1-lutein; 2-zeaxanthin; 3-β-cryptoxanthin; 4-β-carotene; 5-α-carotene; 6-canary xanthophylls A; 7- canary xanthophylls B; 8- canary xanthophylls C; 9- canary xanthophylls D; 10-3'dehydrolutein; 11-2', 3'-anhydrolutein; 12-astaxanthin; 13- α -doradexanthin; 14-adonirubin (phoenicoxanthin): 15-canthaxanthin; 16-3-

hydroxy-echinenone; 17-echinenon; 18-rhodoxantin; 19-4-oxo-rubixanthin; 20-4-oxo-gazaniaxanthin; 21-papilioerythrinone; 22-7,8-dihydrolutein; 23-7, 8, 7', 8'-tetrahydro-zeaxanthin; 24-7,8-dehydro-β-cryptoxanthin.

a) Astaxanthin and β-carotene were found in captive birds only.
b) Only captive birds were studied.
c) Accompanied by an unidentified carotenoid.
d) Canary xanthophylls are the naturally occurring yellow feather pigments, but by adding the berries of an introduced honeysuckle to their diet, waxwings obtain rhodoxanthin that gives feathers their orange coloration.
e) Carotenoids are only found in red feathers. Yellow border is colored by melanin.
f) Yellow plumage is not created by the presence of carotenoid or melanin pigments, but by an as-of-yet unidentified type of pigment.
g) Red plumage gets its color from the means by which the canary xanthophylls are bound to feather keratin.

The process of carotenoid incorporation into the tegument is thought to begin by passive lipid diffusion into maturing cells, such as the skin keratinocytes and feather follicles (Lucas and Stettenheim, 1972). These lipids acumulate in the cell in lipoidal droplets that ultimately provide nourishment as well as lipophilic pigments to the growing feather germ (Menon and Menon, 2000).

There are several physiological parameters that may influence carotenoid accumulation in the body and thus colour intensity. These include: absorbtion of carotenoids in the gut, transport of carotenoids bound to lipoproteins through bloodstream, uptake of circulating carotenoids by maturing feathers, and metabolism of carotenoids at feather follicles. So far, there is evidence in birds that both carotenoid aquisition and certain aspectsof carotenoid utilisation (e.g., lipo-protein production) can regulate color expression among individuals within a given species, but more studies are needed to discern the strength and ubiquity of nutritional and physiological effects on carotenoid colour across bird taxa (Hill and McGraw, 2006).

Concerning the colour differences of the integument or integumentary structures in different regions of the bird's body, the studies showed that this is the responsability of certain binding proteins that shuttle carotenoids intracellularly to be incorporated into specific tissues, at specific times (Brush, 1990; McGraw *et al.*, 2003).

The mechanism for the colour change has yet to be determined and may involve feather soiling or wear, pigment degradation, or feather degradation by bacteria (Shawkey and Hill, 2004). McGraw *et al.*, (2003b) showed that beyond the spatial and temporal specificity of carotenoid accumulation in avian integumentary tissues, these tissues can also preferentially target certain carotenoids.

The intensity of colour induced by carotenoids in birds (both in males and females) has very deep influences on the vitality and surviving capacity of the individuals, on reproductive behavior (choosing the mate before laying the eggs), and also on the quality, value of the eggs, and finally on some characteristics of the offsprings. Nowadays, it is known that biochemical strategies for developing sexually

selected carotenoid coloration can follow one of two alternate routes: the accumulation of as many types and amounts of carotenoids as possible, or the preferential accumulation of one or a few particularly chromatic forms. But, to understand all these aspects concerning the ornamental colours in birds, first, we have to mention some very important roles of carotenoids, more exactly antioxidant and immune functions.

Carotenoids are known as "sacrificial" antioxidants. In other words, carotenoid molecules are not regenerated like other antioxidants, and are degraded in the process of neutralizing free radicals or reactive oxygen species. (Tsuchiya *et al.*, 1994). Thus, carotenoids are capable to protect DNA, proteins and lipids from oxididation, and thereby guaranteing antioxidant protection of the cell membranes and antibodies (Edge *et al.*, 1997). These functions can be noticed and are very important both at the level of adults which take these carotenoids from the diet, and at the level of the yolk, and embryonic tissues, where the rapid cell division, and the rate of metabolic events induce a high level of oxidative stress. This oxidative stress continue a rather long time at the level of offspringt, too (Surai *et al.*, 2001). Since the ability of carotenoids to quench singlet oxygen increases with their maximum absorbtion wavelength, redder carotenoids may provide a more efficient antioxidant defence (Edge *et al.*, 1997).

In vitro studies have revealed that free radical trapping activity is most efficient when certain combinations of antioxidants are involved (Bohm *et al.*, 1997) or within certain rangers of carotenoid concentration and partial oxygen pressure (Burton and Ingold, 1984). Domestic hens caracteristically have an antioxidant activity which depends on the type of carotenoids in their diet correlated with their interaction with antioxidant vitamines. But, so far it is not known yet the way in which these interactions affect the levels and proportions of carotenoids, and also their activity in the yolk (Tengerdy *et al.*, 1990).

Transfer of the antioxidant carotenoids from the mother to the yolk and then to the specific embryo tissues are extremely important becauce, finally these carotenoids confer an antioxidant and immune protection to the offsprings (Young and Lowe, 2001; Blount *et al.*, 2002; Surai, 2002). Metabolic activity of the birds' embryos is very intensive and produce a large number of free radicals. Besides, embryo tissues contain a high level of unsaturated lipids which are extremely susceptible to the oxidative damages. All these aspects request the antioxidant intervention of the carotenoids during this period of life in birds (Surai *et al.*, 2001).

Most of the recent attention to the mechanisms and functions of internal-tissue carotenoids in birds has been placed on developing young, particularly in such precocial species. In their earliest stages of development, embryos are exposed to and assimilate high concentrations of carotenoids in yolk that represent nearly 50% of their mothers' liver stores. Neonates transfer maternally derived carotenoids from yolk to their tissues during development and retain high levels at and after hatching; the liver is the most carotenoid-enriched tissue of newly hatched individuals (Surai *et al.*, 2001). Liver-carotenoid levels are known to rapidly decline within 2 weeks

post-hatch, but some researches has estimated that nearly one-quarter of all liver carotenoids in a 4-week-old chick are still from the yolk (Koustos *et al.*, 2003a). These maternal carotenoids have been showed to protect cells from oxidative damage. Thus, mothers can adaptively boost offspring protection from free radicals (for a long period of time) by allocating carotenoids to yolk (Blount *et al.*, 2000).

Carotenoids guarantee the antioxidant protection of the ovules, in females, but, high levels of carotenoids at the level of testes and semen, in males, demonstrate that they are also involved in antioxidant protection of the spermatozoa, too.(Blount *et al.*, 2001; Hill and McGraw, 2006).

Comparative studies concerning the effects of carotenoids at the level of the males and females are insufficient, but it seems that the lack of these compounds is more important for the last ones. Maternal supplies of liophilic antioxidants can be limiting for egg production in birds (Lin *et al.*, 2002; Surai, 2002; Blount *et al.*, 2004). The quality of the eggs seems to be seriously affected by the carrotenoid in diet. Antioxidant supply may also limit sperm quality, because sperm are particularly susceptible to oxidative damage (Blount *et al.*, 2001; Peters *et al.*, 2004). But, this aspect requests more studies, because, for example, in *Taeniopygia guttata* a lack of carotenoids seems not to affect spermatogenesis at all (Birkhead *et al.*, 1999).

Immunostimulatory function is another extremely important function of carotenoids in birds. Most of the attention to the antioxidant role of carotenoids has centred on their protection of the immune system, due to the inactivation of free radicals produced during immune-cell activation and proliferation (Moller *et al.*, 2000; Hughes, 2001). Thymus, bursa and spleen are now recognized as important carotenoid reservoirs in young birds (Koustos *et al.*, 2003a and b). In young chicks, levels of carotenoids in these tissues are differentially sensitive to dietary and/or maternal carotenoid sources. (Koustos *et al.*, 2003a) and to the immune demands of individuals (Kousta, 2003b). For example, thymic carotenoid levels responded to the dietary intake in dose-dependent fashion, whereas bursal carotenoids did not. Instead, bursal carotenoids were proportionally and highest when dietary carotenoids are low, suggesting that bursa receives priority in carotenoid accumulation when dietary provisions are low (Koustos *et al.*, 2003 b).

In *Larus fuscus*, experimentally, it could be noticed that a dietary carotenoid supply enhances the plasmatic antioxidant activity and immunde defence. These birds have a decreased level of immunoglobulines, which theoretically, could guarantee a better health, which could allow a better feeding which would be better for the individuals themselves, and for their offspring (Blount *et al.*, 2002a).

Lozano (1994) noticed that birds may face a trade-off when allocating carotenoids acquired from the diet to physiological and coloration purposes, and only the highest-quality individuals (those who aquire the most carotenoids or are in the best health-state) can devote more to the integument for advertisement.

One hypothesis for why females, in many bird species, frequently prefer to mate with the most elaborately ornamented males predicts that availability of

carotenoid pigments is a potentially limiting factort for both ornament expression and immune function. An implicit assumption of this hypothesis is that males that can afford to produce more elaborate carotenoid-dependent displays must be healthier individuals, with superior immunocompetence. However, whether variation in circulating carotenoid levels causes variation in both immune function and sexual atractiveness has not been determined in any species, the studies of Blount *et al.* (2003) showed that manipulation (improving) of dietary carotenoid supply invokes parallel changes in cell-mediated immune function and sexual atractiveness in male zebra finches *(Taeniopygia guttata).*

Recent correlational and experimental studies provide some support for the notion that carotenoid colours honestly signal immune capacity, but this notion is by no means universally accepted, and more work is needed on free-ranging birds to better understand carotenoid allocation to body maintanance versus sexual ornamentation. Moreover, depending on the type or concentration of carotenoids, these molecules can also have positive gene-regulatory effects on immune function (e.g., via gap-junctional communication) or detrimental pro-oxidant effects on cells and tissues in the body (Hill and McGraw, 2006).

Carotenoid pigments (modified or native) **are deposited into egg yolk** by female birds, but little is known of the ecological regulation of this process. Some supplies of carotenoids deposited into yolk could have been derived from body stores (body fat, liver, integument) rather than metbolic transformation. However, the generality of this potential mechanism is uncertain, because body stores of carotenoids are insufficient to meet the demands of egg production in domestic hens, and a decline in female integument pigmentation at the time of egg production has been reported in various wild bird species (Burley *et al.*, 1992; Blount *et al.*, 2002a).

Alternatively, the profile of carotenoids deposited into yolk may directly reflect the relative proportions of carotenoids in the maternal diet, although this correlation is not always available (Partali *et al.*, 1987). But, an experimental approach is required to better understand the relatioship between diet and yolk carotenoid composition in wild birds.

Unfortunately, so far, the relative importance of pigment aquisition and utilization at the level of the egg yolk (i.e. physiological discrimination: differential uptake, transport, deposition or metabolic conversions) is poorly understood.

Nowadays, the qualitative differences between the carotenoid profile of egg yolk are intensively investigate (Verboven *et al.*, 2005). Egg development and deposition of different carotenoids at the level of the yolk expend a lot of energy. Aquisition, maternal selective uptake, transport, deposition or metabolic transformation of ingested carotenoids regulate the pattern of yolk enrichment are far less than completely efficient. (Hill, 2000; Blount *et al.*, 2002; 2004; Hill *et al.*, 2002).

At least two proximate sources of between female variation in egg quality can be hypothesised. First, egg quality could be determined by female quality. Maternal age, mass and size generally explain statistically significant, but small

proportions of the variation in egg size (Christian, 2002). The concentration of yolk carotenoids has been shown to be affected by the infection status of the mother (Saino *et al.*, 2002), and to covary with her carotenoid-based integument coloration (Blount *et al.*, 2002). Second, environmental effects such as food supply (Blount *et al.*, 2002) or ambient temperature (Saino *et al.*, 2004) may also influence egg quality, tough results for egg size are inconsistent (Christians, 2002).

There are a lot of significant differences between domestic hens and wild birds concerning the transfer from the diet into the egg yolk. For example, domestic hens characteristically transfer certain carotenoids from the maternal diet into eggs more efficiently than others and can metabolically transform certain carotenoids before deposition into yolk (Hencken, 1992). Domestic hens seemed to asimilate cantaxanthine more efficiently than zeaxanthine (Hencken, 1992), while in *Larus fuscus* the transfer of beta-carotene into the yolk is extremely efficient (Blount *et al.*, 2002a). This explains partially the differences between proportions of carotenoids in yolk.

Differential allocation in relation to the attractiveness of the male partner may be another very important cause of variation among females in egg quality (Burley, 1986). Males which are more intense carotenoid-based coloration have superior foraging success for carotenoids (Hill, 2002), and work harder to provision mates or nestlings (Linville *et al.*, 1998; Senar *et al.*, 2002). For example, a cross fostering experiment using blue tits (*Cyanistes caeruleus)*, showed that chick growth was related to the plumage yellowness of the foster father but not to that of the genetic parent (Senar *et al.*, 2002). Correlation studies in *Cardinalis cardinalis* have shown that individuals with greater carotenoid pigmentation work harder at nestlings (Linville *et al.*, 1998). Therefore, it is worth the female investing more in current reproduction if her mate is attractive, because the value of offspring will be higher, although, sometimes it could be notice some exceptions from this rule (Sheldon, 2000).

Studies in *Cyanistes caeruleus* showed that body size, age and **carotenoid-based plumage coloration may** also **be used by females as indicators of male quality**. Indeed, larger and older males have more success in siring extra-pair young. (Delhey *et al.*, 2003). As for carotenoid-based coloration, in *Parus* species, the main source of carotenoids are caterpillars, thus a brightly carotenoid-coloured individual may signal its ability to find these caterpillars. (Partali *et al.*, 1987).

Besides, at *Cyanistes coeruleus*, the yolk colour was correlated with laying date and yolk mass. Though the effect of laying date could not be explain by ambient temperature during egg formation, it suggests a proximate constraint of general carotenoid availability on yolk composition (Szigeti *et al.*, 2007).

It seems that factors predicting egg size were different from those predicting yolk colour. Carotenoid content is not necessarly proportionate to egg size, suggests that maternal effects occurring during egg production can not be estimated only by egg size (Royle *et al.*, 2003; Szigeti *et al.*, 2007).

Effects of poor neonatal nutrition on adult reproductive performance were studied by Blount *et al.* (2006) who noticed that this kind of nutrition is associated with

reduced blood antioxidant levels in adulthood, which could impair reproductive performance. These effects could be a consequence of an alterated uptake and transport of the antioxidants in diet (Blount *et al.*, 2003), or these are known as limiting factors for the fecundity (Surai, 2002; Blount *et al.*, 2003; Blount *et al.*, 2004).

Besides birds fed with a low quality diet took longer to initiate egg-laying, and then laid eggs at a slower rate, aspect correlated with a reduced breeding success owing to increased hatching asiynchrony and therefore chick mortality. In addition, these birds did not show reduced clutch mass or size, or yolk antioxidant levels. All these reproductive perturbations could be correlated with an irreversible alteration of the morphology, physiology and metabolism which appear at the level of any organism which are lack by carotenoids during the critical period of their development (Lindstrom, 1999; Metcalfe and Monagham, 2001).

In birds, experimentally increased egg production can reduce maternal condition, parenting ability and survival, and the quality of the eggs themselves. Such costs probably reflect resource limitation, but the identity of the resource(s) in question remains unclear. Trade-offs in the allocation of limiting carotenoids between somatic maintenance and egg production could therefore be an important factor underlying reproductive costs. However, whether carotenoids are limiting for egg production directly, by stimulating the synthesis or antioxidant protection of yolk precursors, or indirectly, via effects on maternal health, requires futher study. So far, researches demonstrated clearly that carotenoids are antioxidants and immunomodulants of the adult organism (Blount *et al.*, 2003), and also are involved in egg production, maternally derived carotenoids in eggs yolk enhancing egg quality (Blount *et al.*, 2002a and b).

In birds, clutch loss through predation is common, therefore individuals often must face the decision of whether to relay within a short time-scale. Therefore, Blount *et al.* (2004) tested the capacity of carotenoid-supplemented females to replace a lost clutch in Larus fuscus. These researches demonstrated that carotenoid supplementation increased the capacity of gulls to replace a lost clutch of eggs, one-third more carotenoid-diet females re-laid compared with controls (probably as a consequence of an increased synthesis and/or antioxidant protection of yolk precursors). But, carotenoid supplementation did not influence the latency to re-lay eggs, mass of replacement clutches, clutch size, while the yolk and egg mass covaried proportionately.

If **carotenoids enhance the capacity to lay**, why then did not carotenoid-diet females lay larger replacement eggs? Carotenoid supplementation clearly influenced replacement egg composition: yolk mass and egg mass covaried proportionately in the carotenoid-diet group, whereas in the control-diet group, yolk mass declined with increasing egg mass. Decreasing yolk size with increasing egg size is typical in seabirds, so variation in egg size largely reflects changes in albumen (i.e. protein and water) content (Williams, 1994). Possibly, therefore, some carotenoid-diet re-layers had relatively low supplies of such macronutrients. This stands to reason because, under natural feeding conditions, carotenoids can only be cosumed with macronutrients

– indeed, tissue carotenoids are often bound to lipids or proteins (Olso and Owens, 1998), so maternal levels of carotenoids and macronutrients should covary.

Analysis of clutch replacement in relation to maternal body condition and plasma carotenoid levels suggested that the latter was a stronger determinant (Blount *et al.*, 2004). It cannot exclude some alternative explanations. If carotenoids enhanced the effiency of immune defences, this could have resulted in an increased availability of other limiting nutrients. It is also conceivable that a healthier individual should have better foraging efficiency. These explanations predict that sustained carotenoid supplementation should result in increased maternal body condition, and possibly larger eggs and better as quality, although sudies of Blount *et al.* (2002b si 2004) didn not demonstrate these effects. All these results obtained in *Larus fuscus* species, showed that even for this species which were investigated very intensively to understand better the relative importance of carotenoids requires supplimentary ecophysiological investigations.

So far, no study has been able to clarify whether a greater capacity for work in carotenoid-rich males reflects condition dependence in exercise performance as influenced by carotenoids or alternatively is genetically determined. One possibility is that **carotenoid availability may be limiting for exercise performances**, because of the role of antioxidants in protecting muscles against oxidative damages (Powers *et al.*, 2004).

Recently, Blount and Matheson (2006) tried to establish in laboratory, whether a diet supplemented with additional carotenoids may improve physical (muscle's) performances in *Taeniopygia guttata.* Their results demonstrated that carotenoid supplementation enhanced flight performance, the birds having a shorter flight times than controls, and, besides, less often required a repeat stimulus to elicit escape flight, andemeged sooner from the release chamber after a startle stimulus. Such effects of carotenoids could be ecologically important, since flight take-off performance is thought to be an important determinant of predator evasion and foraging, and thus, of survival probability and the capacity to provide parental care.

Moller *et al.* (2000) hypothesized that birds may be particularly susceptible to carotenoid limitation compared with other taxa because of a high requirement for and turnover of carotenoids. On average, birds typically have far higher blood concentrations of carotenoids than mammals do (Hill, 1999). This is not simply because of differences in carotenoid levels in diet (Slifka *et al.*, 1999), and therefore suggests that birds have a relatively greater need for carotenoids and hence have efficient carotenoid absorbtion and transport systems. The rate of carotenoid uptake and turnover in birds can be high, bodily levels increasing rapidly in response to dietary supplementation (Blount *et al.*, 2003b; McGraw and Ardia, 2003), and declining rapidly in response to immune sensitization (Faivre *et al.*, 2003; McGraw and Ardia, 2003; Alonso-Alvarez *et al.*, 2004). Therefore, the specialists hypothesize that birds' flight muscles may be susceptible to antioxidant limitation, with negative consequence for exercise performance.(Blount and Matheson, 2006). To confirm that dietary carotenoid supplementation resulted in enhanced muscle

resistance to oxidative damage would require that birds be killed for in vitro measurement of tissue oxidation products in muscle. (Coombes *et al.*, 2002).

It is very important to know the possibility that the supplemental carotenoid dose in diet elevates blood carotenoid levels above naturally occuring levels. Unfurtunately, so far, the normal range for blood carotenoids in wild birds are unknown at this time, there are just a few data concerning some captive species when they get a basal diet or a carotenoid supplemented diet. For example, one of the most studied species in laboratory conditions is *Taeniopygia guttata*, in which, it could be noticed that blood carotenoid levels continue to rise in response to increasing dietary carotenoid intake until they reach certain level, after this level of carotenoids in diet, the level of blood carotenoids wouldnt rise at all, but remain constant (McGraw *et al.*, 2003).

Clearly there are several explanations for how carotenoids may influence muscles' performance. Indeed a combination of antioxidant and nonantioxidant mechanisms could be responsible. It has also consider the possibility that carotenoid supplementation, through effects on immune defences, could have resulted in birds being less debilitated by parasites or diseases and hence more capable of flight.

Clearance of *Mycoplasma gallicepticum* bacterium after experimental inoculation has been shown to be faster in male house finches (*Carpodactus mexicanus*) with redder carotenoid-based plumage (Hill and Farmer, 2005). Similary experiments were done in *Taeniopygia guttata,* too (Blount *et al.*, 2003b; McGraw and Ardia, 2003), obtaining similary results. A correct estimate of the immune defence of carotenoids needs that before starting the experiments, to be able to estimate correctly the fitness and the pathogens, parasites or diseases of every individual we are working on. But this is extremely difficult or, in practical terms, perhaps imposible, but it will be necessary to find a solution.

Recent researches demonstrated another very important role of carotenoids – **photoprotection of some tissues, structures.** For example, the macular region of retina is enriched with xantophylls (Khachiket *et al.*, 2002), that arguably play an important role in absorbing harmful short-wave light rays, protecting photoreceptors from oxidative damage, and ultimately preventing age-related macular degeneration (Mozaffarich *et al.*, 2003).in birds with carotenoid-colored eyes such as chickens, ducks, birds may be signaling the direct photoprotection they are affording themselves with their retinal pigments. So far, it is unknown wether species of bird with more colorful carotenoid-based integuments deposit more in their eyes, and what consequences this concentration has for the visual system. By definition, based on the light absorbance properties of the pigments, this process would leave the visual systems of carotenoid-coloured species optimally tuned to accept and perceive the light wavelengths that their integuments reflects (Hill and McGraw, 2006).

One last and recently discovered biological property of carotenoids is their **role in gap-junctional communication** (Tapiero *et al.*, 2004). One method of cell-to-cell signaling (e.g., the exchange of ions, electric currents, or nutrients) is accomplished by the transfer of information via gap junctions – pores that connect the cytosol of two

neighboring cells. Proteins known as " connexins" are the governing body of gap-junctional comunnication, and recent studies have showed that carotenoids can bind to nuclear receptors in cells and upregulate gene expression and the production of connexins. This aspects is very important for the prevention of different types of cancer, in case of which, the gap-junctional comunnication is involved in the cellular malignant transformation (Livny *et al.*, 2002).

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===SHORT COMMUNICATION===

ATMOSPHERIC POLLEN SEASON OF *PLANTAGO* IN TIMIŞOARA

NICOLETA IANOVICI[1]

SUMMARY. The quantitative monitoring of the airborne pollen offers important information regarding the flowering phenophase duration, the possibility of creating pollenic calendars for a certain geographic region and gives hints on the periods when pneumallergens may reach dangerous quantities. This study was planned in order to determine annual dissemination of *Plantago* pollen grains in the atmosphere of Timişoara in 2000-2004. Pollen belonging to *Plantago* was sampled during a 5-year atmospheric pollen-monitoring programme in Timişoara, România, using a VPPS 2000 Lanzoni trap. Annual variations in the concentration of pollen in the atmosphere were analysed by the volumetric method. *Plantago* airpollen constitutes between 0.9% and 3.39% of the annual total of pollen grains. During the studied period, inter-annual variations, concerning the total annual pollen counts and the beginning, peak and ending dates of the Atmospheric Pollen Season (APS), were reported. The daily quantities of pollen are expressed as numbers of pollen grains per cubic meter of air per day (PG/m^3).

Keywords: Atmospheric Pollen Season, *Plantago*

Introduction

The present study constitutes the first attempt to record *Plantago* airborne pollen grains in a qualitative and quantitative way in Timişoara, România.

Allergic diseases represent an increasing problem in the western world, with symptoms that may not be easily distinguished from other disorders (Julge *et al.*, 1998; Bjorksten *et al*., 1999; Platts-Mills *et al*., 2000; von Mutius, 2000; Söderström *et al*., 2003). Furthermore, there are many different allergens, which may trigger the clinical symptoms and it is often not easy to distinguish which allergen that is the most offending one (Yunginger *et al.*, 2000). The study of fluctuations and trends in quantities of pollen in the atmosphere is of importance in various respects. One of the phenomena that might be reflected in the annual totals of daily airborne pollen concentrations is evidence of global change in the climate (Menzel, 2000), the hypothesis being that higher yearly average temperatures will lead to more pollen of some taxa in the atmosphere. Another aspect of interest in quantitative studies of atmospheric pollen is the possible relationship with the observed increase in the prevalence of pollen allergy in the human population in many parts of the world

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(D'Amato *et al.* 1998; Crane *et al*., 2002). Reliable assessment of fluctuations and trends in airborne pollen can only be made by using long-term observations, preferably at several locations (Emberlin *et al*., 2000). Some authors maintain that the effect of climatic change could cause an increase in the production of airborne allergens, and therefore an increase in the severity of symptoms and in the number of people becoming sensitized (Sánchez-Mesa *et al*., 2005). Another factor for the increasing sensitization to pollen in recent years might be the worsening of pollution experienced in the city (Cariñanos et al., 2002), mainly as a consequence of the rising number of dieselengined cars. Within the city of Córdoba, the highest incidence of pollen allergy occurs in the eastern and southern zones. These are the areas containing the bulk of the industrial sites. They also constitute the main routes in and out of the city, where there are frequent traffic jams (Sánchez-Mesa *et al*., 2005). This would support the relationship already observed between pollution and a higher incidence of allergy (Knox *et al*., 1997).

Pollen of *Plantago lanceolata* (English plantain, ribwort) is known to be a common cause of pollinosis in the temperate zones of North America, Australia and Europe, where it is widely distributed. Although the clinical importance of this dicotyledon weed has been largely demonstrated in several studies carried out in different countries (Lewis & Imber, 1975; Spieksma *et al*., 1980; D'Amato & Lobefalo, 1989; Peat, 1991; Subiza *et al*., 1995; Calabonzo *et al.*, 2001), its role in the aetiology of pollinosis has usually been overlooked in clinical practice. This is probably because plantain pollinates in the same season as other relevant allergenic plants, such as grasses, and monosensitization to plantain pollen is not frequently found among allergic patients. Most authors agree that it is difficult to evaluate the true importance of *Plantago* in polynosis symptoms due to the low rate of monosensitized patients and the fact that those allergic to *Plantago* are usually also allergic to *Gramineae* and other airborne pollen types at the same time of the year. As the peak of pollination occurs in June, the allergic symptoms can be mistakenly associated with grass pollen allergy (Gutierrez *et al.*, 1999).

Materials and Methods

The monitoring station within the Department of Biology of the West University, the only one of this kind in Romania, uses a trap VPPS 2000 Lanzoni to aspirate air. The mashine allows the evaluation of airborne pollen dinamics from the city as well as it's surroundings. The results are significant for plain zone from west and south-west România. Analysis of the pollen count and pollen fall distribution was performed on the basis of the data collected in Timişoara in the seasons of 2000–2004. Monitoring station is placed in urban area. Vegetation in the town and its surroundings consists of ruderal vegetation, forests, semi-natural community of grasses and antropomorphic habitats. The volumetric measurement point was located in the Timişoara city at an elevation of 20 m above ground level. The pollen count was expressed as the number of pollen grains in 1 m^3 per 24 h

(Mandrioli *et al.*, 1998). The trap was calibrated weekly to maintain a flow rate of 10 l/min. Pollen was caught on a 24 mm wide transparent tape coated by a thin film of silicon oil. The tape was mounted on a cylinder rotating at a speed of 2 mm per hour. A complete rotation of the cylinder took seven days. Pollen grains were identified on the surface of 4 horizontal bands. Pollen grains identification was carried out using a ML-4M microscope, magnifying 400X. The numbers of pollen grains found in the cover-glass area were converted to pollen counts. The data for 2002 is not complete because of temporary technical problems with the trap.

In this paper, we determined the APS (Atmospheric Pollen Season) in accordance with the criteria used by the following authors: Nilsson and Persson (corresponding to 90% of the total pollen catch -the 90% method), Andersen and Torben (corresponding to 95% of the total pollen catch- the 95% method) (Jato *et al*., 2006).

The morphology of genus *Plantago* pollen suggests that it is stenopalynous; its pollen is spherical, secondarily apolar, small- or medium-sized (D = 19-35 μm), pantoporate, with 5-16 simple operculate pores (D pore = 3-5 μm), which are sometimes surrounded by a 2-2.5 μm ring or annulus. The surface is generally scabrate or verrucate. The main differences at species level are size, greater or lesser number of pores, presence or absence of ring, and the external relief of the exine. Remarkable annulate and non-annulate pollen forms were enumerated for *Plantago* and successfully utilized in taxonomic clarification of the genus (Ubera *et al*., 1988).

Results and Discussions

It is clear that the Romanian pollen seasons show 3 main parts: tree season (February–April), grass season (May–July), weed season (July–October) (Ianovici, 2007). Like other summer weeds, *Plantago* is unlikely to be important, but may contribute to the problems of the pollen sensitive patient under exceptional circumstances (Spieksma *et al.*, 1980).

The pollen shedding time usually occurs from May to the beginning of September. The pollen pattern shows successive peaks taking place from mid May to mid July, but *Plantago* pollen may frequently be found in air samples even when the flowering season is over. The peaks (2 or 3 or several) alternate with days of low pollen concentrations. Every year shows a different pattern with different numbers and periods of the peaks. The annual sums of the daily pollen concentrations and other aerobiological parameters in the years from 2000 to 2004 are given in table 1. Monthly variations of total pollen grains recorded in the atmosphere of Timişoara during the years 2000-2004 are shown in table 2. The mean value of the annual sums is 435.2 PG, with a lowest total of 148 in 2001, and a highest annual total of 669 in 2002. The percentage of the *Plantago* annual sum with respect to the total annual sum is rather low, ranging from 0.9% in 2001 to 3.39% in 2002, with a mean value of 2.28 %. The mean annual concentration for 5 years of study was 435.2 PG.

Table 1.

Most important data characterizing the Atmospheric Pollen Season of Plantago

	2000	**2001**	**2002**	**2003**	**2004**
The starting data of the *Plantago* pollen season	29 May	28May	1 May	1 May	1 May
The ending data of the *Plantago* pollen season	25 August	2 September	18 August	31 July	13 September
Number of days, when was *Plantago* pollen in the air	89	97	110	76	136
Atmospheric Pollen Season (Nilsson & Persson, 1981); 90%	73	78	91	54	95
Atmospheric Pollen Season (Andersen & Torben, 1991); 95%	80	89	96	61	109
Pollen Index	1.72%	0.9%	3.39%	2.6%	2.8%
Days with pollen concentrations higher than 30 PG/m^3	-	-	1	3	-
The peak days in PG/m^3	8	6	30	42	22

Table 2.

Monthly pattern of Plantago airborne pollen (%), Timişoara, România

Year	II	III	IV	V	VI	VII	VIII	IX	X	*annual total of pollen grains*
2004	0	0	0	28%	28,4%	27%	14,7%	1,9%	0	486
2003	0	0	0	39,73%	55,86%	4,41%	39,73%	0	0	657
2002	0	0	0	51.6%	17%	24.7%	6.7%	0	0	669
2001	0	0	0	4%	26.4%	49.3%	19.6%	0.7%	0	148
2000	0	0	0	2.3%	52.8%	29.6%	15.3%	0	0	216

The months in which maximum pollen concentrations were recorded were the same in all five years: May, June and July. The longest Atmospheric Pollen Season was observed in the year 2004 (95 days according to Nilsson & Persson, 1981; 109 days according to Andersen and Torben, 1991). Five year average pollen season duration were 78.2 days (the 90% method) and 87 days (the 95% method). From year 2003, the end of season shows a trend towards earlier dates, resulting in a shorter length of APS. The starts of these APS were fairly uniform. The pollen season started between the beginning and end of May and finished by August depending on the year.

The highest daily concentration is 42 PG/m^3 in 2003. For Timişoara was characteristic lower average number of days with pollen concentrations higher than 30 PG/m^3 (part of season when *Plantago* pollen reached critical concentrations – one day in 2002 and three days in 2003). Overall, the pollen shedding course of the *Plantago* in Timişoara corresponds to that already described during the pollen season in other European areas.

Pollen grains registered in the aeroplankton of the measurement site come from the plants growing in the neighbourhood as well as areas several kilometers

distant. Apart from that, in the atmosphere there is the distant transport pollen, which appears prior or following the period of flowering, or occurs at high concentrations at night (Comtois,1997). The determination of the share of the pollen from distant areas in the aeroplankton is difficult, yet one may presume that the high concentrations of *Plantago* pollen noted in Timişoara on September 2001 and 2004 come from distant transport as they occurred in the pollen season with a significant delay. The concentration of plantain pollen in air reaches low to moderate values (Puc, 2003).

Conclusions

Results of this study demonstrate that *Plantago* seasons occurred at regular intervals between May and August each year; however, individual daily and seasonal *Plantago* counts were heterogeneous. The highest concentration of pollen from *Plantago* taxa occurred in 2002.The length of pollen seasons was calculated by the 95% method and 90% method. Registered data confirm the fact that at least quantitatively *Plantago* pollen is not an important allergenic factor.

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===REVIEW===

ECOLOGICAL AND GENETICAL IMPLICATIONS OF CLINE EVOLUTION

MANUELA DORDEA[1] AND NICOLAE COMAN[1]

SUMMARY. As the environment changes permanently, and migrations occur recurrently, natural selection acts powerfully on population gene pools. More often, the gene flow hinders the action of natural selection. However, natural selection and migration are more often in a steady-state, that assure the specificity of local population gene pools, the clines being a result of such equilibrium, thus proving finally the evolution.

Keywords: Allen's rule, Bergmann's rule, Cope's rule, cline, gene pool, Gloger's rule

Clines in human populations

The human species, *Homo sapiens sapiens*, consists of many populations. Some live in grassland areas, while others in mountains over 4000 m high, in tropical forest areas or desert oasis. Each population is largely adapted to the specific conditions of the regions they have lived in for generations. Under similar conditions, natural selection leads frequently to nearly identical modifications. Thus, in the populations from both the Kalahari and Gobi deserts, the fold, a remainder of the third eyelid, is better developed than in other populations. Tuaregs, living in the Saharan desert, have the melting point of myelin higher than populations from temperate areas, ensuring a greater resistance to high temperatures. The Amerindian females from the Anza Mountains are fertile at elevations higher than 4000 m, unlike those living in grassland areas. The females of different Eskimo tribes have no ovulation, menstrual cycle respectively, during the long polar night.

From the wide range of characteristics of human populations, let us examine the intensity of skin pigmentation and the variation of eye color, starting from the African equatorial zone to the frozen waters of the Arctic Ocean.

At the equator, especially in Bantu populations, the skin and eye pigmentation is very strong. The intensity lowers gradually as one moves away from the equator, so that in the Scandinavian populations white skin, less pigmented and light-colored eyes prevail. Under the equatorial sun beans, the great amount of pigments in skin and eyes protect the human body against the high ultraviolet rays, which might cause severe burns of the skin and blindness.

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In Scandinavian countries, where the solar radiation is diminished, the low skin pigmentation ensures the penetration of a sufficient amount of UV rays to allow vitamin D synthesis D and prevent ricketts.

In the far north of Europe, snow strongly reflects the solar radiation, which may bring about blinding and skin burning. Consequently, natural selection has favoured a stronger pigmentation of skin and eyes, specific to Eskimo populations.

The old European populations, so-called autochthonous or native populations, have been overlapped in time by other populations coming from Asia and north-western Europe. Migratory populations with other gene frequencies have mixed, more or less, with the autochthonous ones.

Regardless of the intensity of miscellany, natural selection has favoured genes that ensure a better survival capacity. One might practically suppose that hybridization between natives and immigrants occurred in all instances. The modifications of the resulting gene pools depended on the differences of gene frequencies between natives and immigrants, on the proportion of immigrants, the frequency of immigration process and, last but not least, on the intensity with which selection favours or not the varied characters or phenotypes, respectively. The more favoured a phenotype is, the more rapidly the gene frequencies which determine that phenotype rise, and conversely. Thus, each local population is under the pressure of natural selection, which influences gene frequency, gene pool respectively, depending on the environment conditions in which that population lives. There are also phenotypes with a high genetic determination, but less under environmental pressure, such as blood groups' phenotypes, ABO or MN. In this case, gene frequencies change very slowly. For example, the blood group B allele frequencies across Eurasia have resulted from the Mongol and Tartar invasions during the 5^{th} – 15^{th} centuries AD. Prior to this, the blood group B allele was presumed to be absent in western Europeans before the Mongol-Tartar migrations. The very low frequency of the blood group B allele in native Basques in Spain and other western European populations support this assumption (Fig. 1).

Therefore, if the migration rates are low and selection is strong, there will be local adaptations. Conversely, when migration rates are high and the effect of selection on phenotypes with strong genetic determination (namely with heritability nearly 1) is weak, clines do not form (cline – gradual modifications of gene frequencies depending on different environmental factors).

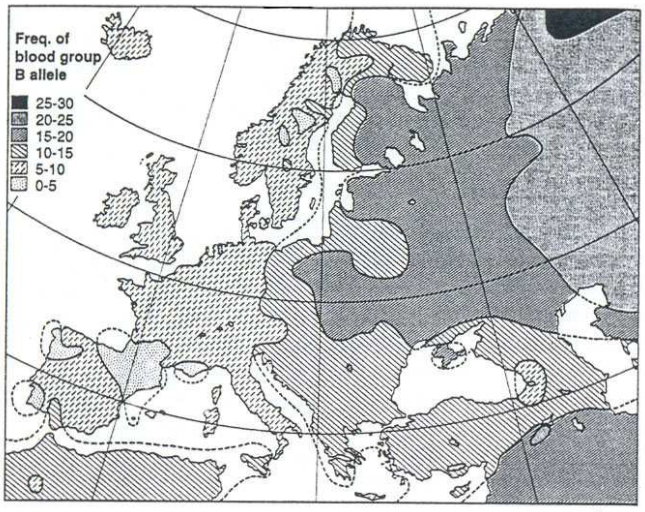

Fig.1. B group allele frequencies across Eurasia, resulting from the Mongol and Tartar invasions between AD 500 and 1500 (after Mourant *et al.*, 1976)

Clinal evolution in *Drosophila*

When the same selection pressure acts upon deleterious mutations all over the distribution area, regardless of the environmental conditions of various local populations, their frequency decreases gradually. This is the case of the frequency of inversions in the five longest chromosomes of *Drosophila subobscura* (Sperlich, 1973). As one can see (fig.2), in the centre of the species distribution area, namely around the Mediterranean Sea, the frequency of the heterozygous inversions is high. It decreases gradually to the borders of the distribution area, so that populations from Iran, Holland, Norway, Scotland have a very low frequency of such inversions. One might conclude that, starting from the centre of the distribution area to the borders, a continuous flow of genetic variability occurs due to migration. By acting in the opposite direction, natural selection maintains the frequency clines.

A lot of latitudinal clines in inversion frequencies in *Drosophila melanogaster* across different continents have been identified by Knibb *et al.* (1981). Hoffmann *et al.* (2004) emphasized that the great chromosomal inversions are involved in the adaptative divergences between conspecific populations.

Studies regarding clines in *Drosophilidae* reveal that they affect a great number of morpho-physiological traits, namely the body size (James *et al.*, 1995), eggs size (Azevedo *et al.*, 1996), rate of larval development (James and Partridge, 1995), wing shape (Hoffmann and Shirriffs, 2002) etc. The influence of thermal variations and photoperiodicity on the eggs size, the length of the eggs laying and the development rate was also noticed in *Drosophila melanogaster* (Coman and Stentzer, 1981).

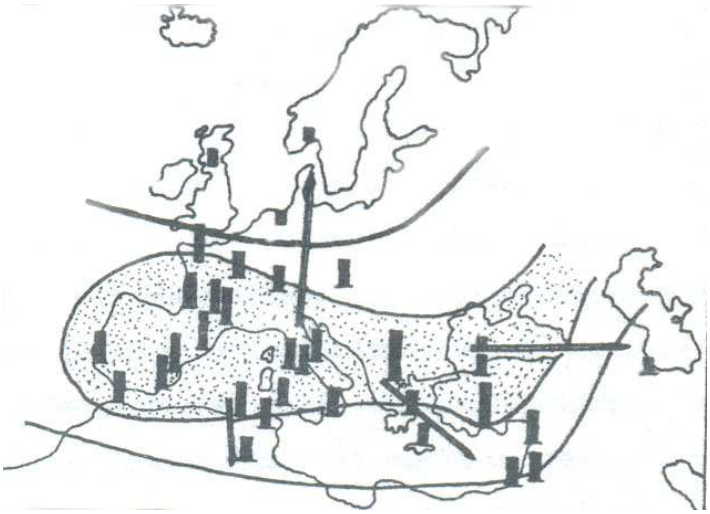

Fig. 2. The frequency of heterozygous inversions as a measure of genetic diversity in *Drosophila subobscura*. The hatched zone represents the central part of the area. The arrows show the direction of migration.

During the last years, researches regarding the mechanisms of cline evolution implied molecular approaches.

In *Drosophila melanogaster* a *shsp* (heat shock genes) family consisting of seven genes located closely together on chromosome 3 at position 67B was detected (Frydenberg *et al.*, 2003). Some of the genes of *shsp* family showed a latitudinal cline in the populations of this species along the eastern coast of Australia.

Referring to the same populations, Anderson *et al.* (2005) described a number of inversions associated with a latitudinal cline in the last 20 years. The cosmopolitan *In*(3R)*Payne* inversion polymorphism of *Drosophila melanogaster* has become of increasing interest because of its potential association with quantitative traits that vary clinally in an adaptative manner. The *In* (3R) *Payne* inversions have high frequency in tropical populations, while relatively rare in the temperate populations of the distribution area. This inversion polymorphism has been associated with quantitative traits with clinal variation, such as body size (Calboli *et al.*, 2003), as well as with thermal resistance traits (Anderson *et al.*, 2003), particularly via one of the heat shock genes, *hsr-omega*.

In the same population of *Drosophila melanogaster* from eastern Australia, Rako *et al.* (2007) reveal strong associations between the clinal chromosomal inversion *In*(3R)*Payne* and markers within it, as well as among these markers. Of the five predicted associations between markers and traits, four were detected (increased heat, decreased cold resistance and body size with the heat shock gene *hsr-omega*, increased cold resistance with the inversion *In*(3R)*Payne*.

It is argued that adult size clines, inversion frequency clines, and clines in allele frequency at loci involved in glycolysis and glycogen storage are part of the same adaptative strategy. De Jong and Bochdanovits (2003) stated that at high latitudes, selection on *Drosophila melanogaster* would favour high larval growth rate at low temperatures, and resource storage in adults to survive winter. On the contrary, at low latitudes selection would favour lower larval critical size to survive crowding, and increased male activity leading to high male reproductive success.

Drosophila melanogaster adults from equatorial Kenya are smaller than flies from Cluj, Romania (personal observations), Denmark or Netherlands (Bochdanovits and De Jong, 2003).

In Australia a cline in body size is present along the coast, from Queensland to Tasmania (James *et al.*, 1995). A similar latitudinal cline in body size was noticed in natural *Drosophila melanogaster* populations from the Baltic to Central Asia, with larger-sized flies at cooler temperatures (Imasheva *et al.*, 1994).

Temperate populations are also larger than tropical populations in wing/thorax ratio. A larger ratio translates into a better flying capacity implying temperate populations would be better flyers. The larger body size in temperate *Drosophila melanogaster* populations has various causes, either a cline in cell size, cell number or in development time. Temperate populations often develop faster or grow faster than tropical ones (James and Partridge, 1995).

De Jong and Bochdanovits (2003) stated that larger body size in temperate *Drosophila melanogaster* populations could be related also with high insulin level, which would lead to relatively high storage of nutrients, which would be necessary for winter survival, higher fecundity and high egg laying in cool spring. A relatively high insulin level would however lead to lower longevity. A relatively low insulin level would lead to lower storage of nutrients, but higher starvation resistance of larvae, and small body size. Low insulin activity promotes longevity rather than fecundity, and male longevity might be important in sexual selection.

Recently, Sambucetti *et al.* (2006) described a clinal variation of developmental time and several size-related traits in *Drosophila buzatii* along an altitudinal gradient from northwestern Argentina, spanning more than 2000 m in hight. Several fitness-related traits (developmental time, thorax length, wing length and wing loading) were measured in two laboratory generations (G7 and G33). The authors concluded that developmental time was positively correlated with the altitude of the origin population. Wing loading tended to be larger in highland than in lowland populations, suggesting that flight performance was subject to stronger selection pressure in highland populations.

In *Drosophila buzatii* latitudinal clines of wing loading in eastern Australia were also noticed (Loeschcke *et al.*, 2000). Geographic variation was also evident in populations of *Drosophila serrata* along a transect on the eastern coast of Australia. In this species the size and wing shapes show a gradual increase with latitudes (Hoffmann and Schirriffs, 2002).

Huey *et al.* (2000) studied the speed of clinal evolution in *Drosophila subobscura*, a species accidentally introduced from Europe into western North and South America in 1978, which spread rapidly in temperate regions. The introduced species, that colonized large areas, was not followed by migration, so clines could quickly evolve. The fly is native to Europe, where it exhibits a clinal increase in wing size with latitude, especially in females. In North American populations, no wing length cline was detected one decade after its introduction. After two decades, however, a cline has evolved and largely converged on the ancestral clines.

Laws of clinal evoution

A typical clinal variation was also evident in Japan, in Emma field cricket (*Teleogryllus emma*), regarding the duration of nymphal development and the width of the adult head in males (Fig. 3). Both increase clinally from north to south (Ricklefs and Miller,1997). Similar observations were recorded in many other species. Generalizing all the available observations, zoologists have stated some laws, which rule clinal variability in animals. Thus, **Bergmann's rule** states that in vertebrates the individuals living at high latitudes tend to be larger than individuals of close relatives living nearer the equator.

Allen's rule refers to the size of body extremities like ears, limbs, or tails, which appear to be smaller in animals living in areas near poles, than they are in their relatives living in warm regions.

Gloger's rule asserts that color intensity diminishes in conspecific populations as one moves away from the equator.

Cope's rule states that population lineages tend to increase body size over geological time (Coman, 2004).

It is noteworthy that all these rules referring to clinal evolution have come under sustained criticism, on account of many exceptions.

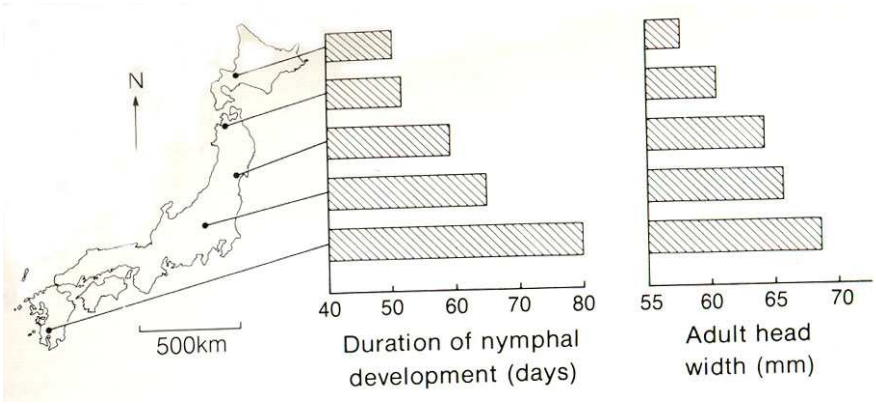

Fig. 3. Clinal geographic variation in duration of nymphal development and the width of the adult head in males from six local strains of the Emma field cricket (*Teleogryllus emma*) in Japan (after Ricklefs and Miller, 1999).

The most debated was **Bergmann's rule**, dated from 1847, which was thought to be valuable only for warm-blooded vertebrates (birds and mammals). In order to test the validity of Bergmann's rule in *Carnivora*, Meiri *et al.* (2004) examined patterns of correlation between skull length and geographical latitude. Only species for which there were more than 20 specimens of a given sex were analyzed. Meiri *et al.* (2004) examined 67 species that comprise 27% of the 244 species of wild carnivores in the order (Nowak, 1999). The authors have found a positive correlation in 50% of carnivore species, which means species that follow Bergmann's rule, significant negative correlation in 11% of species, and not significant correlation in the others.

If warm-blooded animals (birds and mammals) obey Bergmann's rule with some exceptions, poikilotherm species rarely conform to the rule.

Ashton and Feldman (2003) evaluated the relation between body size and latitude in order to verify if Bergmann's rule hold true for reptiles: chelonians (turtles) and squamates (lizards and snakes). They have found that in chelonians 19 of 23 species increase in size with latitude, whereas squamates followed the converse to Bergmann's rule (61 of 83 species decrease in size with latitude). In cooler areas, the lizards and snakes with smaller body size gain heat more rapidly, which means in fact a clinal variation also, which is not conform with Bergmann's rule.

A general explanation for latitudinal and thermal clines in body size will reside in the context of life-history theory. A relatively large body size in cooler environment might be the result of a decrease of competition or advantage in obtaining food.

Angilletta *et al.* (2004) consider that in lizards, the mechanism responsible for larger body sizes in colder environments is delayed maturation, which results in a greater fecundity, a lower survival capacity to maturity. Higher survivorship of juveniles in colder environments can favour the evolution of Bergmann's cline. Therefore, Bergmann's clines result from interactions between biotic and abiotic factors, which create an equilibrium between costs and benefits (Angilletta *et al.*, 2004).

Laugen *et al.* (2005) studied the validity of Bergmann's rule in populations of common frog (*Rana temporaria*) across a 1600 km long latitudinal gradient in Scandinavia, both for wild collected adults and laboratory-reared individuals. The authors found that in adults, the mean body size increased from south to mid-latitudes, and declined thereafter. This occurred despite the fact that the mean age of adult frogs increased with increasing latitude, and age and body size were positively correlated. Their results suggest that the pattern of body size variation across the latitudinal cline might be at least partly genetically determined, and that although there is a considerable geographic variation in the mean body size of *Rana temporaria*, this variation does not conform to Bergmann's rule. The latitudinal pattern of body size variation in individuals reared in laboratory was similar.

Ashton (2002) suggested that precipitation and humidity could be important selective factors behind latitudinal and altitudinal body size trends in

adult amphibians. Since large individuals have better desiccation tolerance than smaller individuals, this could select for large body sizes in dry environments.

In Bocas del Toro Archipelago of Panama, Summers *et al.* (2003) quantified the levels of variation in spectral reflectance within and among populations of *Dendrobates pumilio*, generally confirming Gloger's rule. The authors suggested the possibility of a sexual selection for clinal variation in coloration among the studied populations.

Similarly to Bergmann's rule, **Cope's rule**, illustrated with the evolution of horse' families, *Equidae*, has many exceptions. In order to clarify the mechanisms of clinal evolution illustrated by Bergmann's rule, Cope's rule, Rapoports'rule, Ashton (2001) recommended that meta-analytical techniques, a group of statistical methods rarely used in ecology and evolution, need to be incorporated in future tests of general trends.

Clinal variation in plants

Clinal variation were also noticed in populations of different species of grasses (*Anthoxanthum odoratum*, *Festuca ovine*) which have heavy metal tolerance, allowing them to colonize polluted slagheaps from old mines in Wales (Briggs and Walters, 1997). Certainly, the heavy metal tolerance is the result of mutations that accidentally appeared in the genofonds of that species. Only species with genes for heavy metal tolerance succeeded in colonizing mine wastes in Great Britain (Briggs and Walters, 1997).

Clausen *et al.* (1941) noticed a clinal variation in *Potentilla glandulosa* as early as 1941. They found remarkable morphologic differences in conspecific populations growing at different elevations (30, 1530 and 2330m, respectively). Such morphological differences were also noticed between populations of Edelweiss (*Leontopodium alpine*), growing on the alpine slopes of the Fagaras and the Bucegi mountains and those at low altitudes at Intregalde, near Aiud. The average size of plants growing at high altitudes is small; the flowers are big and covered with white wooly hairs. The specimens growing on hayfield at Intregalde are two times taller. Their flowers are small and the hairs on flowers and leaves scarce (personal observations).

At Gurahont, near Arad, *Ilex aquifolium* (holly) has the northern limit of distribution. The species survive against the winter frost of the region protected under the high crown of beech forests. Under such circumstances, its height is nearly 3 m, rarely 5 m. The evergreen leaves are thinner and not so leathery as compared to the specimens growing in the Mediterranean zone (personal observations).

The baobab (*Adansonia digitata*) also called the monkey-bread tree, a huge tree of tropical Africa, makes fruits annually in Guinea and Sudan savannas. In regions with low rainfalls, such as in Sahel savannas, the trees grow only along temporary rivers (weds). Here, they are more precocious and their fruits much smaller. In very droughty years, even if the trees survive, their fruits do not reach maturity (Coman, 1975).

Campbell and Reece (2005) describe a clinal variation in populations of yarrow plants (*Achillea borealis*) growing on the slopes of the Sierra Nevada Mountains. The mean height of plants collected from 1000 m altitude was 80 cm. The average plant sizes gradually decrease with increasing elevation, so that at 3500 m altitude, plants were only 20 cm height (Fig. 4).

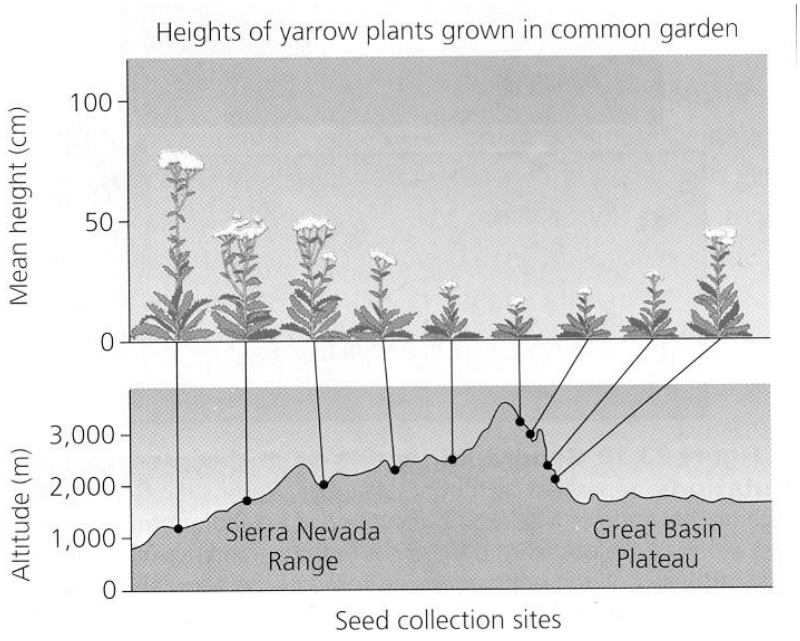

Fig. 4. Clinal variation in yarrow plants (*Achillea borealis*) growing on the slopes of the Sierra Mountains

Jolivet and Bernasconi (2007) have recently reported a clinal variation in the European populations of the white campion (*Silene latifolia*). The authors investigated six geographically separated European populations of the white campion, both for molecular variation at six newly developed microsatellite loci and for quantitative variation in morphological and life-history traits (age at first flowering, plant size, number of leaves, number of stems, time of germination, etc.). The age at first flowering increased significantly with latitude. Based on the clinal variation for the age at first flowering observed in S, *latifolia*, the authors suggested that the species is adapted to local environments by its phenology and morphology, having a strong adaptative potential. One of the possible explanations of this clinal variation is the particularly interesting biotic interaction between *S. latifolia* and the specialist seed predator *Hadena bicruris* (Jolivet and Bernasconi, 2006; Wolfe *et al.*, 2004).

Mølmann *et al.* (2006) describe an interesting case of clinal variability in Norway spruce (*Picea abies*). Seedlings of these trees ceased growth when night-

lengths become shorter than a critical value, and this critical night-length decreased clinally with increasing latitude of origin. The light quality also appeared to play an important role in clinal variation. Northern populations required higher irradiance of both far-red and red light than southern populations. Far-red and red light were more effective in maintaining growth than blue light. The authors suggested that phytochrome(s) are the primary photoreceptors in high irradiance responses maintaining growth in Norway spruce seedlings.

Similar responses to different light qualities, documenting a clinal increase in requirement for far-red light with increasing latitude were noticed for downy birch (*Betula pubescens*) and bay willow (Håbjørg, 1972; Juntilla, 1976).

Color pattern variation in island populations

Clinal variation was found also in populations of bull trout (*Salvelinus confluentus*), native in northwestern North America. There are differences between subpopulations that reside in high mountain streams throughout life, and the ones that reside in large rivers and lakes (Dordea and Coman, 2005). Human activities, especially logging, mining, dam- and road-building have drastically reduced the possibility of migration between subpopulations of bull trout (Campbell *et al.*, 1999).

Clinal evolution of color pattern in the northern water snake *Nerodia* (*Natrix*) *sipedon* was for a long time debated. As we already mentioned in a previous paper (Dordea and Coman, 2006) Camin and Ehrlich (1958) initiated the studies regarding the coloration of these snakes in the area of Lake Erie, United States. Close to the shore of Lake Erie, there are small islands with rocky and sandy beaches. As far back as 1937, Conant and Clay noticed that snakes swim from the shores of the lake to islands for breeding. This assumption was later confirmed by Camin and Ehrlich (1958) who concluded that the effect of migration of banded snakes from mainland to islands reduce the effect of natural selection on islands favouring the unbanded individuals. The genetic mechanism responsible for the coloration pattern in water snake is not yet well clear. However, King (1993a) assumed that a complex of genes are involved in the color pattern, the ones that determine bandation being dominant.

Mainland populations of water snakes consist solely of banded individuals. On islands, the snake populations exhibit a continuous variation in color pattern, ranging from regularly banded to uniformly grey, unbanded individuals, the latter ones prevailing. On the mainland with heavily vegetated marshland, natural selection favours the banded water snakes, which offer more protection against predators, especially sea birds.

On the contrary, on islands the frequency of banded individuals is lowering from young ones to adults. Obviously, the unbanded individuals are better adapted to islands where streams are missing and individuals have to cross the sandy beaches to reach the water sources. Moreover, predators, especially sea birds,

easier hunt the banded individuals. Thus, this cryptic coloration increases snakes survivorship on islands (King, 1993b).

Later on, mark-recapture methods confirmed the selective advantage of unbanded individuals on islands. The camouflage of unbanded forms was efficient especially during the first year of life, when the frequency of banded juveniles was high (46%) (Camin and Ehrlich, 1958). The permanent flow of banded individuals from the mainland to islands, prevent island populations to have only unbanded coloration pattern (King and Lawson, 1995). In conclusion, the color pattern variation in island populations results from a balance between gene flow and natural selection.

The differences of gene frequency between conspecific clines are more evident as the capacity of individuals' movement is lower (Haliburton, 2004). Such differences were reported in industrial melanic butterflies (Bishop and Cook, 1975).

General conclusions

Frankham *et al.* (2002) consider that plants, which are subjected not only to climatic changes but also to changes in soil composition, exhibit frequency clines more accentuated than animals. The fact that plants do not move, except for pollination and different techniques of seed dissemination, certainly accounts for this increased cline frequency.

Using simulation models, Kelly (2006) confirmed the former conclusions.

As the environment changes permanently, and migrations occur recurrently, natural selection acts powerfully on population gene pools. More often, the gene flow hinders the action of natural selection. However, natural selection and migration are more often in a steady-state, that assure the specificity of local population gene pools, the clines being a result of such equilibrium, thus proving finally the evolution.

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===REVIEW===

IRON METABOLISM DISORDER EXPLORATION: CLASSICAL AND MODERN PARAMETERS

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SUMMARY. The biochemical serum markers commonly used to investigate iron metabolism proved to be effective means in solving many of the diagnostic problems raised by the various clinical settings encountered in practice. However, other investigations – hematological ones, assessing the hemoglobinization of red cells – are needed for real-time differentiation between anemia of chronic disease with and without functional iron deficiency. Combining the biochemical and hematological parameters into a single diagram (the so-called diagnostic&therapeutic plot) provides yet another valuable diagnostic tool, useful also in therapy. The recently discovered liver hormone hepcidin appears to be the central regulator of iron metabolism. Other proteins, as well as certain genes, involved in iron metabolism have also been discovered lately, some of them with diagnostic or therapeutic potential.

Keywords: iron metabolism, biochemical markers, functional iron deficiency, reticulocyte hemoglobin content, percentage of hypochromic erythrocytes, diagnostic and therapeutic plot, immature reticulocyte fraction, hepcidin.

Introduction

Iron is one of the most widespread elements in nature. It is encountered everywhere in the living world playing important roles in the living organisms: oxygen and electron transporter participating in respiration and energy production; catalyst of several essential metabolic processes, DNA synthesis and cellular proliferation; detoxifying role by reducing the oxidative stress (Hershko,1995). On the other hand, however, iron is itself capable of producing- because of the easy transition between its oxidation states (ferrous and ferric)-reactive species of oxygen (free radicals), having thus a harmful potential for the body (Hinzmann, 2003). As a result both deficiency and excess of iron can have severe pathological consequences, mainly anemias and iron overload (hemochromatosis), respectively (Brittenham, 1991). Therefore, mechanisms exist that strictly regulate iron metabolism, maintaining the balance of iron in the body (Weiss, 1999). Normally, only minimal losses of iron occur, iron being preserved in the body and reused again and again. There exists an iron cycle in the body that consists , essentially, of the one-way flow of iron from the circulation to the erythrocyte-producing bone marrow, then to the circulating red blood cells and, by the aging of these cells, to the reticuloendothelial system, from where the iron gets back into the circulation and the cycle recommences (Lee and Herbert, 1998). Iron

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homeostasis regulation is unipolar, performed exclusively at the level of entry of iron in the body; there is no regulation by loss, losses of greater amounts of iron –through blood loss- occurring only in pathological conditions (Brittenham, 1991).

The intestinal absorption of iron is controlled by at least three independent mechanisms: dietary- a higher iron intake renders the intestinal cells refractory for a while to the absorption of iron; the size of the iron stores in the body; and the level of the marrow erythropoietic activity (Andrews, 1999).

Serum iron measurement. For a long time, serum iron measurement held a central position in the investigation of iron metabolism (Rymer, 1996) and continues to be used very often even today although its sensitivity in detecting iron deficiency is rather low (Koss, 1998). However, it may still be useful sometimes in differentiating the hypochromic anemias when the other tests are inconclusive. The iron measured is iron bound to serum transferrin and does not include hemoglobin iron. The ferric iron is removed from transferrin, transformed to the ferrous state by the addition of a chromogenic reagent, resulting a colored complex that can be measured spectrophotometrically. The assay is analytically very sensitive. Hemolysis and anticoagulation falsify the results. Morning samples of blood have to be used, fasting for 12 hours and any iron-containing medication not being taken for 12-24 hours preceding the test. Serum iron levels show diurnal variation, being about 25% lower in the evening. The reference range varies with age and sex and also, slightly, with the testing method used. Normal values: **60-175 microg/dL** for men, **50-170 microg/dL** for women. Low values are encountered in iron deficiency anemia and anemia of chronic diseases, high ones in sideroblastic anemias and hemochromatosis (Koss, 1998).

Total iron binding capacity (TIBC) measures the amount of iron that the circulating transferrin could bind in a fully saturated state (normally only about 30% of transferrin is bound to iron).It is an indirect measure of transferrin concentration, expressed as an iron measurement. In combination with serum iron it serves to calculate transferrin saturation with iron. Serum iron and TIBC are most useful in the evaluation of chronic iron overload states and acute iron poisonings. The TIBC test consists of adding ferric iron to the sample, removing all excess, unbound iron and analyzing the remaining sample for iron content using the serum iron method described above. Specimen requirements are the same as for serum iron determination; however, unlike serum iron, TIBC values are not dependent on the time of day the sample is drawn (Koss, 1998). The TIBC reference range for adults is **250 to 425 microg/dL**. Serum transferrin concentration may be calculated from the TIBC by using the formula: serum transferrin (in g/L) = 0,007 x TIBC .

Transferrin saturation may be calculated only if serum iron and TIBC values are available. It is usually used to diagnose iron deficiency, but with a poor

specificity because a low saturation may also be seen in anemias of chronic disease. More importantly, high saturations may be indicative of iron overload. Saturation (in percents) is equal to the ratio of serum iron to TIBC (both expressed in microg/dL), multiplied by one hundred. The reference range is **20-50%** saturation in men and **15-50%** saturation in women (Koss, 1998).

Serum ferritin. Ferritin, composed of iron and a protein part called apoferritin, is the main storage form of iron in the body. The extent of the iron stores is reflected by the ferritin found in the serum (Koss, 1998). It was considered until quite recently that each microgram per liter ferritin in the serum would correspond to about 8 mg tissue storage iron, but actually the ferritin level in the serum offers only a guideline, the serum ferritin – storage iron relationship being linear only in a limited value interval (Rymer, 1996). Serum ferritin measurement is valuable in diagnosing iron deficiency because it is, generally, the first laboratory test to become abnormal when iron stores begin to decrease, and before erythrocyte morphology shows any signs of abnormality. Among the hypochromic anemias (iron deficiency anemia, anemia of chronic disease, sideroblastic anemias, minor thalassemia) only iron deficiency anemia shows decreased serum ferritin values, but even increased (or normal) values do not exclude with certainty the presence of iron deficiency, ferritin being an acute phase reactant that increases during inflammatory processes. Serum ferritin can be measured using radioimmunoassays (RIA), enzyme immuno- assays (EIA) and immunoradiometric assays. The reference range varies with method, age and sex. It is **20-250 microg/L** in men and **10-120 microg/L** in women. Its level generally increases in women in menopause and children generally have low ferritin levels, except during the first month of life. In contrast to serum iron, serum ferritin does not have diurnal variations, neither is it influenced by exogenous iron ingestion (Koss, 1998).

Almost all of the circulating ferritin is in a glycosylated form, thus the **glycosylated ferritin** measurement may reveal a pathological cell lysis that releases nonglycosylated ferritin into the circulation and reduces the proportion of glycosylated ferritin in the overall serum ferritin (Rymer, 1996).

The **erythrocyte ferritin** represents the storage form of iron in the erythrocyte prior to its use in the synthesis of hemoglobin. It reflects the balance between the iron entering (brought by transferrin) and leaving (through the hemoglobin synthesis) the cells; the increased entry, unjustified by increased needs (as happens in hemochromatosis) or the faulty utilization (hemoglobinopathies) increase the ferritin in the erythrocyte, while a reduced delivery or an accelerated utilization of iron (as in hemolytic anemias) decrease it. Although these changes are late manifestations of an affected equilibrium between in- and outputs, erythrocyte ferritin has the advantage of not being influenced by inflammatory processes. In iron overloads erythrocyte ferritin shows an outstanding

discriminative capacity, differentiating overload in hemochromatosis from that due to alcohol abuse. Erythrocyte ferritin may be used in the surveillance of treatment through repeated bleedings in hemochromatosis, which must be continued even after the collapse of serum ferritin- the iron overload being still important, until the collapse of the erythrocyte ferritin also takes place, a sign that the treatment has achieved its goal (Rymer, 1996).

To eliminate the influence of red cell volume and packed cell volume (hematocrit), the result obtained at the measurement of erythrocyte ferritin in a hemolysate is divided to the number of erythrocytes present. The normal values are **5-38 attog/erythrocyte** for men and **3-24 attog/erythrocyte** for women. It is worth mentioning the absence of low values in women of fertile age group, as distinct from serum ferritin (Rymer, 1996).

There exist at present attempts to develop an immunological test for the detection of ring sideroblasts in sideroblastic anemias, more specific and more reliable than the conventional Perls reaction that establishes sometimes with difficulty their presence. The test became possible once it was realized that the iron in the granules encircling the nucleus of the ringed sideroblasts is actually in the form of ferritin (the **mitochondrial ferritin**), a protein against which antibodies may be raised, usable thereafter for their detection (Cazzola *et al.*, 2003).

Free erythrocyte protoporphyrin, zinc protoporphyrin. Protoporphyrin IX is the porphyrin compound forming with ferrous iron the heme needed for hemoglobin synthesis. Normally, red cells produce slightly more protoporphyrin than is needed, but when iron is deficient or cannot be properly coupled, protoporphyrin levels build up to several times the normal level as **zinc protoporphyrin (ZPP).** Although **free erythrocyte protoporphyrin (FEP)** generally is a very sensitive and valuable early indicator of an iron metabolism disorder, it is not very specific and cannot be used in differentiating iron deficiency, anemia of chronic diseases and sideroblastic anemias because it may be increased in all these conditions. However, FEP is generally normal in thalassemia, being therefore helpful in differentiating thalassemia from iron deficiency anemia and the anemia of chronic disease.

There is disagreement about which laboratory test is the earliest indicator of the onset of a decrease in iron stores. Some advocate serum ferritin, others the FEP. Anyway, an elevated FEP may draw attention to an abnormal iron utilization and can be used as a screening test.

Using whole blood free erythrocyte porphyrins may be measured by extraction methods. A hematofluorometric method measures only ZPP. ZPP and FEP measurements are not equivalent because extraction methods measure all porphyrins including those bound to zinc, but for practical purposes there is good correlation between them. The reference range depends on the method used. Generally, the range for both FEP and ZPP is **17 to 77 microg/dL erythrocytes**. The reference range for ZPP may also be expressed as **30 to 70 micromol ZPP/mol** of heme (Koss, 1998).

The **soluble transferrin receptor** is a truncated form of the tissue transferrin receptor, present on cells that have a need in iron. Over 80% of the cellular receptors is located in the erythroid marrow, so that the concentration of the circulating soluble receptor is primarily determined by the erythropoietic activity of the marrow. Decreased levels are found in erythroid hypoplasias (aplastic or hypoplastic anemia, chronic renal failure), increased levels in erythroid hyperplasia (thalassemia major, sickle cell anemia, chronic hemolytic anemia), but also in iron deficiency, the lack of iron causing increased receptor synthesis, and the receptors are eventually shed into the blood. In the absence of other conditions causing erythroid hyperplasia, an increase in sTfR concentration provides a sensitive, quantitative measure of tissue iron deficiency. Most importantly, the circulating receptor concentration is not increased in infection or inflammation, unlike ferritin. Immunoassays detecting the soluble transferrin receptor are now available (Brittenham *et al.*, 2000). The reference ranges depend on the commercial kit used, being different for the different companies producing it. The main drawback for its widespread use is the lack of standardization, due primarily to rivalry between manufacturers (Cook *et al.*, 2003, Metzgeroth and Hastka, 2004, Brugnara, 2003).

The contribution of the newly recognized **transferrin receptor 2** to the circulating receptor pool has not yet been established (Brittenham *et al.*, 2000).

The use of soluble transferrin receptor (sTfR) and serum ferritin (F) can help distinguish among iron deficiency anemia (IDA), anemia of chronic disease (ACD), and the combined presence of these by considering the ferritin values lower than 30 ng/ml to be characteristic of IDA, those higher than 100 ng/ml as typical of ACD, while those between 30 and 100 ng/ml may represent either the ACD or the combined ACD&IDA. Differentiation between them is made using the so-called sTfR/F index –that is, the ratio of sTfR to F or, even better, to logF - , the ratio being lower than 1 in the first case (ACD) and greater than 2 in the other (ACD & IDA). Measurement of circulating cytokines, normal in amount in IDA but increased in ACD&IDA, may once more reinforce the diagnosis established as described above (Weiss and Goodnough, 2005).

Functional iron deficiency in ACD, a term used to describe the incapacity of full but blocked iron stores to deliver iron to the marrow (thus when both deficient endogenous EPO and full but blocked iron stores exist), but also the impossibility of iron supply to keep pace with the increased demands (Hinzmann, 2003, Weiss, Goodnough, 2005, Thomas andThomas, 2002, Fitzsimons, Brock, 2001) during exogenous EPO therapy, cannot be diagnosed in real time by the usual biochemical tests. This can be achieved only by hemoglobinization (that is, the hemoglobin content) measurements of red cells (Thomas and Thomas, 2005). Hemoglobin concentration and cell volume of reticulocytes and erythrocytes can be measured flow-cytometrically, cell by cell, based on laser light scattering by the cells; from the product of these parameters the analyzer then calculates automatically the Hb content of the cell. Two new parameters thus result: the **reticulocyte hemoglobin content (CHr)** and the

percentage of **hypochromic erythrocytes (%HYPO).** Hypochromic erythrocytes (%HYPO) (that is, the percentage of erythrocytes with a Hb content less than 28 pg), normally below 2.5%, is increased and CHr is decreased in iron deficiency, irrespective of the presence or absence of inflammation (Hinzmann, 2003) (although some studies indicate the opposite) (Metzgeroth and Hastka, 2004, Hackeng *et al.*, 2004). Since erythrocytes have a lifetime of about 120 days, %HYPO represents a long-time average value, whereas the Hb content of reticulocytes, that exist only for a few days before they mature into erythrocytes, acts like a "snapshot" of the acute condition and is therefore a much more useful tool to immediately assess the success or failure of treatment. Both CHr and the reticulocyte count indicate much earlier than other measurements (hemoglobin, red cell count) the response to treatment, but the former has the advantage of referring also to the functional quality (hemoglobinization) of the cells (Hinzmann, 2003, Metzgeroth and Hastka, 2004).

A so-called diagnostic and therapeutic plot (Fig. 1) has been proposed to assess iron status in ACD and also the evolution of iron deficiency in IDA and ACD with and without functional iron deficiency. The therapeutic implications of the plot are to differentiate patients who should be administered oral iron, exogenous EPO or a combination of EPO and iron (Thomas and Thomas, 2005).

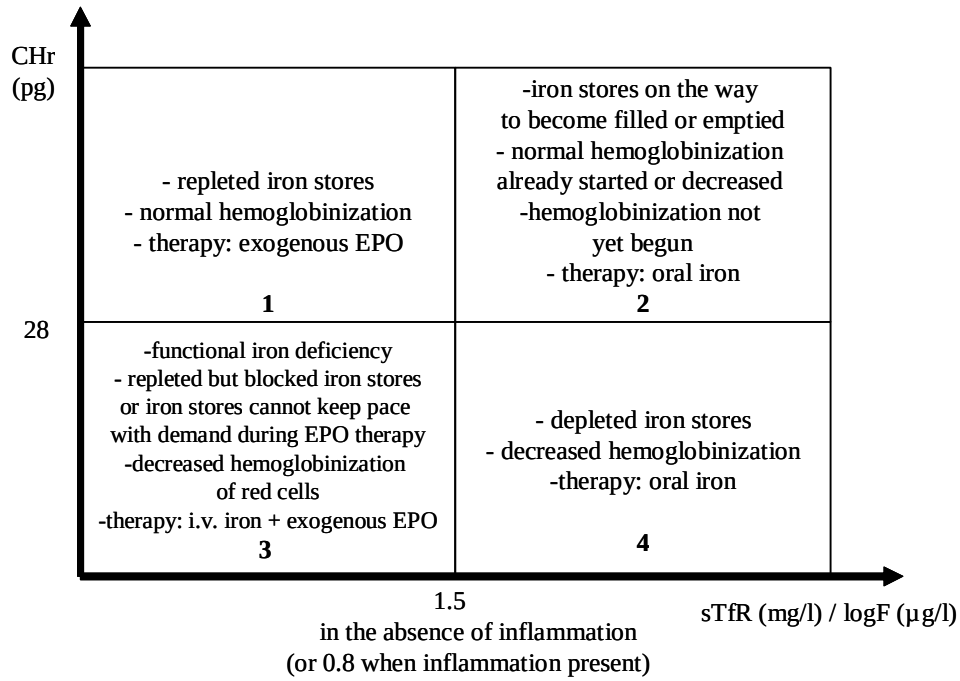

Fig. 1. The diagnostic and therapeutic plot (adapted from Thomas and Thomas, 2002)

The sTfR-F index on the abscissa is plotted against the CHr on the ordinate. Using appropriate cut-off values for both parameters, four areas result: patients in **area 1** have full iron stores and show normal hemoglobinization of red cells. Their anemia is due to a deficiency of endogenous erythropoietin (EPO) in chronic disease, cancer, chronic hemodialysis. Treatment is by exogenous, recombinant EPO, but patients may develop functional iron deficiency due to the incapacity of iron delivery to satisfy the needs imposed by therapy, getting thus into area 4. Patients in **area 3** have IDA with empty iron stores and decreased hemoglobinization of the reticulocytes, requiring oral iron therapy.

Many patients in **area 2** are "coming" from area 3 and are "on the way" to area 1 during iron replenishment. They stay "in transition" in area 2 for four to six weeks. Other patients in area 2 may come from area 1 by developing in the course of their disease a latent iron deficiency with iron stores decreasing but still high enough to ensure normal hemoglobinization of the reticulocytes. Thirdly, those with hyperproliferative erythropoiesis, as after acute hemorrhage, in hemolytic anemia or in late pregnancy, also belong to area 2.

Area 4 (the functional iron deficiency area) represents the patients with chronic disease, cancer or on chronic hemodialysis who have a deficiency of endogenous EPO and full but blocked iron stores, and also the patients in area 1 whose iron needs during therapy with exogenous EPO exceed the iron supply to the marrow. Intravenous iron and exogenous EPO have to be given to them (Hinzmann, 2003, Thomas, Thomas, 2002) .

The beta-thalassemias are, also, predominantly found in area 4(Hinzmann, 2003, Thomas and Thomas, 2002).

The cut-off value for the reticulocyte hemoglobin content is about 28 pg, while that for sTfR/F depends on whether inflammation (which modifies ferritin in the ratio) is present or not: it is 0.8 in the former, 1.5 in the latter case. The C-reactive protein can distinguish between the two situations, being greater than 5 mg/L when inflammation present (Hinzmann, 2003, Metzgeroth Hastka, 2004, Thomas and Thomas, 2002, Brugnara, 2003).

There are other uses of the hematological indices as well: recently developed iron deficiency displays an inverted, that is less than 1, ratio of the hemoglobin content of reticulocytes to hemoglobin content of more mature red cells, as compared with the normal ratio, where the reticulocyte content is greater (Thomas and Thomas, 2002).

Diagnosis of beta-thalassemia trait has been facilitated by determination of the ratio of the percentage of microcytic cells to the percentage of hypochromic red cells, a ratio greater than 0.9 and microcytic red cells more than 20% raising this suspicion. For confirmation, well established methods (electrophoresis, chromatography) may be used (Thomas and Thomas, 2002).

CHr appears to be also in children superior to other markers in predicting iron deficiency and IDA (Hinzmann, 2003).

Using fluorochrome staining and flow cytometry it is possible to quantify the reticulocyte fractions with low-, middle- and high-fluorescence intensity and

thus to assess reticulocyte maturation and to identify the most immature reticulocytes, which contain more RNA and form therefore the high-fluorescence fraction (Metsgeroth and Hastka, 2004, Dotson, 1993).

Measurement of the **immature reticulocyte fraction**, also called the "left-shifted" reticulocyte count, has proved its clinical usefulness in following chemotherapy, EPO therapy and marrow failure (Koss, 1998).

Several proteins, but also some genes, involved at different levels in iron homeostasis and metabolism, have recently been identified: **iron-regulatory proteins** (IRP-1, IRP-2); **HFE protein** – the product of the HFE gene, mutated in hemochromatosis; **divalent metal transporter** (DMT 1); **transferrin receptor 2**; **hephaestin**; the **"Stimulator of Fe Transport "** (SFT); **frataxin**; **ferroportin 1** (Brittenham *et al.*, 2000) .

HFE protein binds to the cellular transferrin receptor and seems to determine receptor affinity for iron-transporting transferrin. The iron-transferrin – transferrin receptor +/-HFE complex enters the cell by internalization within an endosome, then iron is freed and transported across the endosomal membrane via DMT -1, getting into the cytoplasm. In erythroid cells most of the iron passes to the mitochondria and is used for hemoglobin synthesis. The iron-regulatory proteins control iron availability by controlling, at translational level, the synthesis of transferrin receptor (thereby increasing iron uptake by the cell) and of ferritin (increasing iron storage) in the cell. They bind to "iron-responsive elements" in the transferrin-receptor– and ferritin messenger RNA, in the mRNAs for the erythroid-specific delta-ALA synthase and mitochondrial aconitase, as well as in one of the two isoforms of DMT -1. The IRPs thus connect intracellular iron availability with cellular iron utilization, erythropoiesis, mitochondrial energy metabolism and cellular responses to inflammation and oxidative stress, providing a coordinated regulation of these. For example, shortage in iron causes binding of IRP to IRE that inhibits delta-ALA synthase (the first enzyme in the heme synthesis pathway) and ferritin syntheses and TfR mRNA degradation. Conversely, a high iron level in the cell has the opposite effects.

The SFT enhances both transferrin- and nontransferrin-bound iron transport, while frataxin (a mitochondrial protein), expressed in neuronal and cardiac tissue, seems to be involved in mitochondrial iron homeostasis. DMT -1 also serves as a transporter of iron from the intestinal lumen into the enterocyte and hephaestin facilitates the exit of iron from the enterocyte into the circulation (Brittenham *et al.*, 2000).

The outstandingly important role of the liver in determining the amount of iron absorbed from the intestine and released from the storage sites - thus in the modulation of iron release from the cell - mediated by ferroportin 1, becomes more and more evident. The newly discovered liver hormone, **hepcidin**, is considered at present the central regulator of iron homeostasis and metabolism. Factors known to influence intestinal iron absorption, such as the body's iron stores, erythropoietic marrow activity, the blood level of hemoglobin, oxygen and inflammatory cytokines, also regulate liver hepcidin expression. Absorption increases with decreased expression and decreases when expression increases. Enterocytes, the macrophages of the reticuloendothelial

system and liver cells – that is, the cells by which absorption and storage of iron take place – are the cellular targets of hepcidin . Ferroportin accomplishes iron export from these cells, and hepcidin, by binding to ferroportin, causes the internalization and degradation of ferroportin in the cell, thus preventing the exit of iron from the cell. The effects are decreased transfer of iron from enterocytes into the circulation and the increase of iron stores in macrophages and liver cells. Liver cells also possess transferrin receptors of type 2 that may act as a sensor of circulating iron and thereby influence hepcidin expression, hepcidin synthesis being modulated also by HFE protein and the product of a recently discovered gene – **hemojuvelin**. Iron homeostasis regulation by hepcidin offers a unifying explanation for the diametrically opposed abnormalities in iron metabolism seen in hereditary hemochromatosis and the anemia of chronic disease: hepcidin expression is low in the former and increased in the latter condition, with all the consequences deriving from this (Fleming and Bacon, 2005).

The HFE gene is mutated in most of the patients with hereditary hemochromatosis, although the genes coding for transferrin receptor 2 or hemojuvelin are the genes affected in others. The last one is mutated in most persons with juvenile hereditary hemochromatosis (Fleming and Bacon, 2005).

Conclusions

These achievements in the better understanding of the pathophysiological aspects will also have, without doubt, an impact on the diagnosis and therapy of iron metabolism disorders. In some respects the practical benefits are already present: the discovery, in 1996, of the HFE gene that is mutated in most patients with hereditary hemochromatosis has provided a powerful genetic diagnostic test that has radically transformed the diagnostic strategy and overall management of a disease that, from now on, curiously enough, joins the genetic diagnosis with a therapy (venesection) worthy of the Middle Ages (Brittenham *et al.*, 2000) !

Hepcidin antagonists or, on the contrary, exogenous hepcidin may become, in the future, new therapeutic tools in the anemia of chronic disease and hemochromatosis, respectively, likewise to the use of erythropoiesis-stimulating hormones or cytokines in inflammatory conditions (Fleming and Bacon, 2005).

Iron metabolism exploration, which commenced almost a century ago with the measurement of blood iron concentration, has entered the molecular age (Rymer, 1996).

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ANTIMICROBIAL EFFICIENCY OF SOME DISINFECTANTS AGAINST BACTERIA AND FUNGI WITH SURFACE TEST

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SUMMARY. The efficiency of seven disinfectants (Terralin, Domestos, Microzid AF, Omnicide, P3-Topax66, Quick javel, Cidex) was tested against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Bacillus subtilis* ATCC 6633, *Aspergillus niger* ATCC 16404 with surface tests. Surface test were used in order to evaluate the efficiency of disinfectants during the everyday sanitizing practice in the special industrial and hospital surfaces. Test organisms represented pathogen-, spore forming bacteria, yeasts and moulds. Surface test was started with minimal concentration of agents and was not increased above the maximal concentrations which were offered by manufacturers in order not to corrode surfaces or risk the safety of use. Test organisms were inoculated on test areas and after drying inoculated surfaces were treated with disinfectants. Seven disinfectants were tested and four were effective against every test organisms (Omnicide: 0.5 %, P3-Topax66: 5%, Cidex: 100 %, Terralin 0.5 %). Three disinfectants were ineffective against *Aspergillus niger* (Terralin: 0.2 %, Domestos: 2 %, Topax 0.2) and 1 against *Bacillus subrilis* and *Candida albicans* (Microzid AF: 100%).

Keywords: disinfectant, sanitation, bacteria, fungi, surface test

Introduction

The source of microbial contamination in process systems can be the raw material, product-contact surfaces, air and manufacturing workers. In hospitals the source of microbial contamination can be the patients, the nurses and those surfaces and objects which are touched by them. Disinfection is the main tool for controlling contamination from equipment surfaces. The overall aim of sanitation is to prevent microbial growth during the interproduction period (Zottola and Sasahara, 1994). Microorganism can adapt to disinfectants therefore the effectiveness of these cleaning agents must be controlled periodically to minimize acquired resistance. Wilson (1986) reported several methods to select proper concentration of disinfectants for effective microbial control of aseptic processing areas. European Suspension Test (Anonymus, 1993) has been developed to investigate the bactericide, fungicide and sporocide efficiency of disinfectant. Suspension tests do not simulate disinfection on product contact surfaces. Surface tests evaluate efficiency of disinfectants on hard surfaces which are common in food industry, hospitals and demonstrate their activity against microorganisms on contaminated surfaces. Impedimetric detection of the effectiveness of disinfectants has been also developed (Holah et al., 1990). This method provides possibility for investigation of sanitation in suspension and on surfaces as well.

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The aim of this work was to investigate the ability of seven commercial disinfectants against different strains of bacteria and fungi in suspension on different industrial and hospital surfaces.

Materials and methods

*Cultures***.** Test cultures used for investigations were chosen according to Wilson, J. D. (1986) and Pharmacopeial Forum (2002): *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Bacillus subtilis* ATCC 6633, *Aspergillus niger* ATCC 16404. They were obtained from CE Remel (England). Strains were stored on agar slants at 4 °C. *Staphylococcus aureus*, *Escherichia coli* was grown at 36±2°C for 24-48 hours. *Pseudomonas aeruginosa* and *Bacillus subtilis* was incubated at 33±2 °C for 24-48 hours. *Candida albicans* was grown at 23±2 °C, 48-72 hours while *Aspergillus niger was* grown at 23±2 °C for 5 days.

*Sanitizers***.** Seven types of sanitizers were tested. They were diluted according to the manufacturer instructions. Dilution was made with tap-water. The active agent and recommended concentration of sanitizers are listed in Table 1.

Sanitizer treatment. In the first step the maximum of recommended dilution of disinfectants (Terralin 0.2-0.5%, P3-Topax66 2-5%, Domestos 1%, Microzid AF 100 %, Omnicide 0.2-0.5 %, Quick javel 1%, Cidex 1%) were used. Dilution was made with tap-water except Cidex and Microzid AF, which were used without dilution. When the results of first test were not acceptable the concentration of disinfectants was increased and the test was repeated. All tests were repeated twice for each test-organism.

The method was elaborated according to Good Manufacturing Practise (GMP) recommendation, to Pharmacopeial Forum (Anonymus, 2002) and to Wilson (1986). 25 cm^2 test-areas were determined and designated for each disinfectant and for control in glass, stainless steel, tile, plastic, wood.

A dilution of 10^4 organisms per ml was made from each test-culture. Phosphate-buffer was used for dilution. Volume of 0.1 ml of test cultures were spot-inoculated on the determined and designated areas after cleaning with detergent, rinsing with tap-water, degreasing and disinfecting with 70 % ethanol. Each test-area was inoculated with approximately 10^3 test organisms/25 cm^2. The surface was air-dried at room temperature for 30 minutes. After drying 0.2 ml of diluted disinfectants were inoculated in each test-area. Exposure time was 30 minute while disinfected surfaces were dried just like in the industrial and hospital practice. Control-areas were not treated with any disinfectant. Sampling was made with contact media by analogy with common hygienic control practice. Contact media contains a range of neutralizing agents to inactivate the disinfectants. After proper incubation (*Staphylococcus aureus:* 36±2 °C, 24-48 hours; *Escherichia coli:* 36±2 °C, 24-48 hours; *Pseudomonas aeruginosa:* 33±2 °C, 24-48 hours; *Candida albicans:* 23±2 °C, 48-72 hours; *Bacillus subtilis:* 33±2 °C, 48 hours; *Aspergillus niger:* 23±2 °C, 5 days) the number of survived cells (CFU/25 cm^2) was compared to the control.

Table 1.

Disinfectants tested against test-organisms by surface-test

Disinfectant	Recommended concentration	Active agents
P3-Topax66	2-5 %	Potassium-hydroxide, Sodium-hypochlorite, Non-ionic surfactant
Terralin	0.2-0.5 %	Dimethylbenzil – alchil- amoniuclorid, fenoxipropanol, Non-ionic surfactant
Domestos	1-2 %	Sodium-hypochlorite, Non-ionic surfactant, Sodium-hydroxide
Microzid AF	Undiluted	Etanol , Propanol
Omnicide	0.2-0.5 %	Glutaraldehyde, Dimetilbenzil-coco-amoniumclorid
Quick javel	1 %	Sodium- diclorizocianurat, Sodium-carbonat, Adipic acid
Cidex	Undiluted	Glutaraldehyde

The efficiency of disinfectants was evaluated by the formula: $\mathbf{LRV = \log N_0/N}$ where LRV is the logarithmic reduction value of CFU, N_0 is the initial and N is the final number of organisms (CFU/25 cm^2). The efficiency of disinfectants was accepted when: LRV ≥ 2. The data in Tables show the averages of parallel tests. **PRV** (Percentable reduction value of CFU) was used for statistical evaluation which were carried out with MINITAB 9.2 software (ANOVA).

Results and Discussion

Tables 2 and 3 show the effect of Microzid AF on the viability of the six test organisms. There was no significant difference in the efficiency of Microzid AF on the different test surfaces except for the wood surface but there was on different test organisms (α=0.05). Fisher method showed significant difference in the efficiency of Microzid AF against *Bacillus subtilis*, *Aspergillus niger* and *Candida albicans* ascompared to other test organisms. Similarly, Pasanen et al. (1997) found that the effectiveness of alcohol based products to inhibit fungal growth was relatively low. The lower biocidal effect of alcohols, one of the main components of Microzid AF, was not surprising as alcohols are quite ineffective in destroying bacteria and fungi.

P3-Topax 66 was effective against all test organisms in 5% solution. Non-ionic surfactant compounds provide good penetrating ability into the organic materials on surfaces. Hypo-chlorites provide bactericide, fungicide and virucide effect.

The efficiency of P3-Topax is shown in Tables 2 and 3. According to the result of the surface test the 0.2 of P3-Topax was not effective against *Bacillus subtilis* and *Candida albicans.* This fact has to be taken into consideration as this disinfectant is used in food-industry. No significant difference was shown in the efficiency of P3-Topax66 5% on different test surfaces (α=0.05), however, P3-Topax66 5% was significantly more efficient on *E. coli*, *Ps. aeruginosa* and *S. aureus* than *B. subtilis*, *C. albicans* and *A. niger* analysed by Fisher method. Hypo-chlorites are effective against various type of organisms demanding very short contact time (Burge et al. 1989).

Pasanen and co-workers (1997) reported that hypo-chlorite based products provided the best biocidal effect, preventing fungal growth on dusty sheet metal.

After cells were exposed to Omnicide in 0.5% solution the number of test organisms was reduced by at least 2 log cycles on every surface (Table 2, 3). No significant difference was observed comparing different test organisms and surfaces. Good bactericide and fungicide properties of Omnicide are the result of strong antimicrobial action of aldehides. They cause protein and enzyme denaturation and membrane damage of microbial cell.

According to the results of surface test (Tables 2 and 3), Domestos was effective in 1% solution against all test organisms except for *A. niger*. The results of surface test of Borneff et al.(1988) also demonstrate bactericide effect of Domestos. Because Domestos is a chlorine based disinfectant the good sanitizing activity was not surprising. Hypo-chlorites are effective against various types of organisms (Burge et al. 1989). The weak inhibitory effect against *A. niger* was unexpected. In contrast with our results Pasanen et al.(1997) reported that hypo-chlorite based products provided the best biocidal effect, preventing fungal growth completely on dusty sheet metal. In case of *A. niger* increased concentration to 2% was not effective either. The concentration of Domestos was not raised above 2% because a higher concentration of chlorine is deleterious on surfaces during everyday disinfection. This indicates that application of Domestos in the recommended concentration may cause microbiological contamination with this fungi. Non-ionic surfactant compounds provide good penetrating ability into the organic materials on surfaces.

Quick javel, similarly to Domestos, was not effective against *A. niger* (Tables 2 and 3). Results of surface test of P3-Topax66 and Domestos show less effectiveness against *C. albicans* than did Waltimo et al. (1999). They reported that 5 and 0.5 %-of sodium-hipochlorite was effective against the test organisms.

Quaternary ammonium compounds are considered as bound biocides (Burge et al. 1989). They are attached chemically to the surface providing a permanent effective dose during sanitation (Kemper and White, 1991, Speier and Malek, 1982). In spite of this fact the results of Terralin (Table 2 and 3)- with quaternary ammonium compounds as active agent- are similar to the results of Domestos. Both were ineffective against *A. niger* in solution of 0.2% and 1%. Fisher method showed significant difference in the efficiency of Terralin 0.2% against *S. aureus, B. subtilis, C. albicans, A. niger* as compared to other test organisms. In the repeated test increased concentration (0.5) was used, which was effective, the number of test organisms was reduced by at least 2 log cycles on every surface. The investigations of Pasanen et al.(1997) presented similarly low effectiveness of quaterner ammonium compounds against filamentous fungi. Bobichon et al. (1993) demonstrated similar effectiveness of Terralin against *C. albicans* in surface test.

Cidex was the most effective disinfectant (Tables 2 and 3) as the LRV was more than 2 in every test. Cidex was effective against every test organisms. Similarly to Omnicide, the active agent in Cidex is an aldehidic compound (glutaraldehide). Glutaraldehide react with functional groups of the cell (e.g. –COOH, -CHO, $-NH_2$, $-SH_2$, -OH) causing cytotic effect on microbial cells. There was no significant

difference in any surface or against any test organisms. The antifungal effect of Cidex on *C. albicans* were investigated by Yuce et al. (1996) using a rubber germ carrier method. They also observed very good antifungal properties of this sanitizer. Subcultures of *C. albicans* strains showed no growth after 2 and 7 days of incubation.

Conclusions

The results obtained in the present study for the seven studied disinfectants showed that four of them were efficient (Omnicide 0.5%, P3Topax66 5%, Cydex undiluted, Terralin 0.5%) against the studied microorganisms (*Staph. aureus, E. coli, Ps. aeruginosa, Bac. subtilis, C. albicans, Asp. niger*).

The next three disinfectants (Terralin 0.2%, Domestos 2%, P3Topax66) were inefficient against *Asp. niger*, while Microzid AF undiluted was inefficient against *Bac. subtilis* and *Candida albicans.*

Statistical analysis showed no significant difference in the efficiency of disinfectants on the different test surfaces which means that all surfaces which were tested and common in food plants and hospitals are proper and equally easy to clean and disinfect. Although it has to be mentioned that on wooden surface alcohol based biocides are quite ineffective in destruction of fungi.

Table 2.

The logarithmic reduction value of CFU (LRV) using different disinfectants against non-spore forming test bacteria on different surfaces

Microorganism	*E. coli*					*Ps. Aeruginosa*					*Staph. aureus*				
	a	b	c	d	e	a	b	c	d	e	a	b	c	d	e
P3-Topax 66 2 %	2.8	2.3	>2.0	2.0	2.3	>2.0	>2.0	>2.0	2.3	2.2	2.0	>2.0	>2.0	>2.17	>2.0
P3-Topax 66 5 %	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	>2.0	>2.0	>2.3	>2.8	>2.4
Terralin 0.2 %	2.3	1.8	>2.0	>2.0	2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	1.6	>2.0	1.8
Terralin 0.5 %	NT	NT	>2,0	>2,0	>2,0	NT	NT	NT	NT	NT	3,0	3,0	2,6	2,6	2,2
Domestos 1%	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	2.0	>2.0	>2.0	2.5	>2.0	>2.0	>2.0	>2.0	2.7
Domestos 2%	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MicrozidAF100 %	>2.0	>2.0	>2.0	>2.0	2.3	>2.0	2.3	>2.0	2.3	>2.0	3.0	3.0	>2.0	2.1	>2.0
Omnicide 0.2%	>2.0	2.3	2.3	>2.0	>2.0	>2.0	>2.0	>2.0	2.6	2.3	>2.0	2.7	>2.0	>2.0	1.8
Omnicide 0.5%	>2.0	2.3	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	5.0	>2.0
Quick Javel 1%	2.3	>2.0	2.3	2.6	2.6	5.0	2.6	5.0	5.0	5.0	>2.0	>2.0	>2.0	>2.0	>2.0
Cidex	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	2.2	2.7	>2.0	>2.0	2.7

NT means not tested; a= Glass; b= Plastic; c= Stainless steal; d= Tile e= wood

Table 3.

The logarithmic reduction value of CFU (LRV) using different disinfectants against test fungi and *Bac.subtilis* on different surfaces

Microorganism	*C. albicans*					*A. niger*					*B. subtilis*				
	a	b	C	d	e	a	b	c	d	e	a	b	C	d	e
P3-Topax66 2 %	>2.0	2.0	1.8	>2.0	1.6	1.2	1.4	1.6	1.3	1.4	2.2	2.0	2.0	2.4	2.0
P3-Topax66 5 %	2.3	>2.0	>2.0	2.3	2.6	>2.0	1.9	1.6	1.4	1.6	NT	NT	NT	NT	NT
Terralin 0.2 %	>2.0	1.8	1.8	>2.0	2.0	1.0	1.0	1.0	1.0	1.0	>2.0	1.8	1.6	2.0	1.8
Terralin 0.5 %	NT	>2.0	>2.0	NT	NT	2.0	2.0	2.0	1.8	1.8	2.3	2.6	2.4	>2.0	>2.0
Domestos 1%	>2.0	>2.0	2.0	>2.0	2.1	0.4	0.2	0.2	0.4	0.4	NT	NT	NT	NT	NT
Domestos 2%	NT	NT	NT	NT	NT	1.4	0.7	1.3	1.9	1.0	2.2	2.0	2.0	>2.0	>2.0
Microzid AF	>2.0	1.7	1.7	2.0	1.7	1.1	1.9	1.9	1.7	2.3	1.5	1.6	1.8	1.4	1.6
Omnicide 0.2%	1.3	1.5	>2.0	1.8	2.1	2.7	2.7	2.7	2.7	2.4	2.2	1.8	>2.0	2.1	>2.0
Omnicide 0.5	>3.0	>3.0	>3.0	>3.0	2.4	2.7	2.7	>2.	>2.0	2.4	2.2	>3.0	3.0	>2.0	3.0
Quick javel 1%	>2.0	>2.0	>2.0	>2.0	>2.0	>2.0	NT	NT	NT	NT	>2.0	>2.0	>2.0	NT	NT
Cidex	>2.0	>2.0	>2.0	>2.0	2.4	2.7	>2.	2.7	2.7	2.7	>2.0	>2.0	>2.0	2.4	2.0

NT means not tested; a= Glass; b= Plastic; c= Stainless steal; d= Tile; e= Wood,

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BACTERIAL AND ENZYMATIC POTENTIAL OF THE EXPERIMENTAL PLOTS INSTALLED ON THE IRON MINE SPOILS IN IARA (CLUJ COUNTY)

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SUMMARY. The paper presents the evolution of the bacterial and enzymatic potential in the soils of the experimental plots installed on the iron mine spoils in Iara, in May 2005, in the frame of a bioremediation experiment. 26 experimental plots were submitted to different treatments, and sowed with herbaceous species belonging to the families Poaceae (*Festuca rubra, Festuca arundinacea, Dactylis glomerata, Lolium perenne*) and Fabaceae (*Onobrychis viciifolia, Trifolium repens, Trifolium pratense, Lotus corniculatus* and *Medicago sativa*). Microbiological and enzymological analyses were carried out after three months since the experiment had been initiated, and after one year of vegetation. The microbial potential was appreciate according to the bacterial indicators of soil quality (BISQ), calculated taking into account the number of bacteria which belong to the following 5 ecophysiological groups: aerobic mesophilic heterotrophs, ammonifiers, denitrifiers, iron-reducers and sulphate reducing bacteria. The enzymatic potential was appreciate by the enzymatic indicators of soil quality (EISQ), calculated on the base of the phosphatase, catalase, actual and potential dehydrogenase activities. The most efficient treatment applied to the experimental plots proved to be the coverage with a 10-cm layer of soil: the plots covered with soil had higher values of both bacterial and enzymatic indicators of soil quality than the uncovered ones. A remarkable increase of both bacterial, and enzymatic potential has been registered in all the experimental plots in the second year of the experiment. According to the values of both the bacterial, and enzymatic indicators of soil quality after one year of vegetation, one can be stated that the most suited species to be used for bioremediation of such spoils is *Medicago sativa*.

Keywords: mine spoils, bacterial and enzymatic indicators of soil quality, bioremediation

Introduction

The spoils resulted from mining represent an important source of the environmental pollution. The development of mining and other industries results in increasing amounts of wastes. About 1.6×10^{12} m^3 of mine spoils accumulated on the earth up to 1980. This amount increases yearly by about 40×10^9 m^3, approximately three times more than the amount of soil affected yearly by the water erosion (Kiss *et al.*, 1998). According to a report of the Ministry of Waters and Environment Protection, in the year 2000, 251 spoil dumps were registered in Romania, covering a total surface of 5,932 ha. The recultivation of wastelands becomes, first of all a problem of environment protection, as well as a major economic necessity.

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The storage of mine waste material in spoil dumps generates different forms of impact on the environment: damage of large surfaces that can no longer be used for other purposes for a very long time period, spoiling of the natural landscape, with the elimination of some vegetal and animal species, entrainment of stored materials by the torrents formed during abundant rains with negative consequences for downstream localities, pollution of soil, underground and surface waters by various compounds solubilized by the action of meteoric waters, air pollution by the entrainment and transport of fine waste particles at considerable distances by air currents etc.

The bioremediation aims to transform the spoils into technogenic soils, proper for agriculture, forestry or other purposes, like fitting out of parks, sports fields etc. Good results were obtained in the last years by Romanian researchers in recultivation of the spoils resulted from the underground mining of lead and zinc ores in Rodna (Bistrița-Năsăud county): Kiss *et al*., 1989, 1990, 1992; Cristea *et al*., 1995; Paşca *et al*., 1998, 2000; Muntean *et al*., 2001, 2002).

In this paper we present an experiment for bioremediation of the spoils resulted from the underground mining of iron in Iara (Cluj county). The spoil dump in Iara covers approximately 29 ha and has approximately two millions m^3. The evolution of the bacterial and enzymatic potential in the soil of the experimental plots installed on the spoil dump during the first year of experiment is emphasized.

Material and Methods

A biological re-cultivation experiment was initiated on the spoil dump in Iara, in May 2005. Twenty six experimental plots, each of 10 m^2, were installed on the spoil dump, submitted to different treatments, and cultivated with the herbaceous species mentioned in Table 1. The odd plots were covered with a 10-cm layer of soil, and the even plots remained uncovered. Both types of plots were fertilized with 300 g NPK 1:1:1 (300 kg × ha^{-1}) and 200 g NH_4NO_3 (200 kg × ha^{-1}).

The following 5 ecophysiological groups of bacteria have been analyzed: aerobic mesophilic heterotrophs (agar plates) (Atlas, 2004), ammonifiers (peptone medium), denitrifiers (De Barjac culture medium) (Pochon, 1954), iron-reducers (Ottow medium, 1968), and sulphate reducing bacteria (Van Delden medium) (Allen, 1957). Except for the aerobic mesophilic heterotrophs (where the method of successive dilutions, was used), the most probable number of bacteria was calculated according to the statistical table of Alexander (1965). The following four enzymes were studied: phosphatase – activity expressed in mg phenol × 2.5 g^{-1} dry matter soil (Krámer and Erdei, 1959); catalase – activity expressed in mg splitted H_2O_2 × 1.5 g^{-1} dry matter soil (Kappen, 1913); actual and potential dehydrogenase – activities expressed in mg formazan × 2.5 g^{-1} dry matter soil (Casida *et al.*, 1964).

Table 1.

Experimental plots installed on the mine spoil dump in Iara

Experimental plot	Aspect	Terrace	Soil	Species
1	South	II	+	*Festuca rubra*
2			-	
3		III	+	*Onobrychis viciifolia*
4			-	
5		IV	+	*Dactylis glomerata*
6			-	
7		V	+	*Trifolium repens*
8			-	
9		V	+	*Lolium perenne*
10			-	
11		Surface	+	*Lolium perenne* (55%) + *Festuca arundinacea* (40%) + *Festuca rubra* (5%)
12			-	
13		Surface	+	*Festuca rubra* (50%) + *Trifolium pratense* (50%)
14			-	
15	East	II	+	*Dactylis glomerata*
16			-	
17		III	+	*Onobrychis viciifolia*
18			-	
19		IV	+	*Lolium perenne*
20			-	
21		V	+	*Lotus corniculatus*
22			-	
23	North	V	+	*Trifolium pratense*
24			-	
25		Surface	+	*Medicago sativa*
26			-	

Results and Discussion

Preliminary physico-chemical analyses were carried out on the mine spoils in the autumn of 2004, in order to establish the toxic charge of the spoils. The decreasing order of the metal concentration in the raw spoils is: Fe (10^4) > Ca (10^4) > Mg (10^3-10^4) > Cr (10^3-10^4) > Pb (10^2-10^3) > Zn (10^2) > Cu (10^2) > Ni (10^1) (order of magnitude – ppm – in parentheses) (Muntean *et al.*, 2005). The high level of metal concentration explains the toxicity of the raw spoils, the lack of any vegetation, even on the first terrace of 21-years old. No statistically significant difference was registered either among terraces, or among aspects.

The microbiological and enzymological analyses were carried out in August 2005, after three months since the experiment had been initiated, and in August 2006, after one year of vegetation. We analyzed the soils of the 26 experimental plots comparatively with two controls: the raw spoil and a brown luvic soil sampled from a grassland in the next vicinity of the spoil dump. The sampling depth was 30 cm, and the samples of the odd plots, covered with soil, resulted by mixing the upper soil layer (10 cm) with the covered spoil.

The microbiological analyses showed the lack of the sulphate reducing bacteria in all the plots uncovered with soil, both at the beginning of the experiment, and after one year. The sulphate reducing bacteria were present only in 9 experimental plots at the beginning, and in all the 13 experimental plots covered with soil after one year, but their number was the lowest (10^1-10^2 cells × g^{-1} dry matter soil), as compared to the other bacterial groups.

The aerobic mesophilic heterotrophs, ammonifiers, denitrifiers and iron-reducers were present in all the experimental plots. Their number decreases in the order: aerobic mesophilic heterotrophs (10^5-10^6 cells × g^{-1} dry matter soil) > ammonifiers (10^2-10^4 cells × g^{-1} dry matter soil) > denitrifiers and iron-reducers (10^2-10^3 cells × g^{-1} dry matter soil).

As regard the enzymological analyses, we mention that at the beginning of the experiment no actual or potential dehydrogenase activity was detected in the plots uncovered with soil. The two activities were present in all the plots covered with soil, at a level lower than 0.2 mg formazan × 3 g^{-1} dry matter soil. In the second year after revegetation, the actual dehydrogenase activity was also detected only in the experimental plots covered with soil, at a higher intensity, but not overpassing 0.4 mg formazan × 3 g^{-1} dry matter soil. The potential dehydrogenase activity was registered in all the experimental plots, in five of those covered with soil overpassing 1 mg formazan × 3 g^{-1} dry matter soil.

The catalase and phosphatase activities were registered in all the samples analyzed, at higher values in the plots covered with soil, than in the uncovered ones, both at the beginning of the experiment, and after one year of vegetation. The values of both enzymatic activities were higher after one year of vegetation. The maximum values of the phosphatase activity were 1.406 mg phenol × 2.5 g^{-1} dry matter soil (2005, plot 13) and 4.556 mg phenol × 2.5 g^{-1} dry matter soil (2006, plot 23). The most intense catalase activity was 22.436 mg splitted H_2O_2 × 1.5 g^{-1} dry matter soil (2005, plot 11) and 26.592 mg splitted H_2O_2 × 1.5 g^{-1} dry matter soil (2006, plot 15).

The general bacterial potential of the experimental plots was appreciate on the base of the bacterial indicators of soil quality (BISQ) values, calculated taking into account the number of bacteria belonging to each ecophysiological group (Muntean, 1995-1996). The enzymatic indicators of soil quality (BISQ) were also calculated, on the base of the individual value of each activity (Muntean *et al*., 1996). A strong positive correlation, statistically very significant ($p<0.001$) has been established between the bacterial and the enzymatic indicators of soil quality: $r = +0.960$ in 2005, and $r = +0.970$ in 2006.

The results of both microbiological, and enzymological analyses are comparable with those obtained in other similar experiments of mine spoil bioremediation: Kiss *et al*., 1989, 1990, 1992; Cristea *et al*., 1995; Paşca *et al*., 1998, 2000; Muntean *et al*., 2001, 2002).

Fig. 1 presents the evolution of the microbial and enzymatic potential of each experimental plot during the first year of vegetation, as this potential is defined by the values of the bacterial and enzymatic indicator of soil quality.

First of all we can remark the difference between the two controls and the experimental plots, especially after one year of vegetation, as regard the biological

potential. Only in the soil sampled from the vicinity of the spoil dump the BISQ values overpass 5, in both years. In the spoil raw the values of the BISQ are lower than 2.6. Between the experimental plots, the highest value of the BISQ (3.799) was registered after one year of vegetation in the experimental plot 25 (covered with soil and sowed with *Medicago sativa*).

Important differences among the controls and the experimental plots are also registered as regard the enzymatic potential: EISQ = 0.029 – spoil raw in the first year, and EISQ = 0.243 – control soil in the second year. The values of the EISQ can theoretically range from 0 to 1. The highest value of the BISQ (0.137) was registered after one year of vegetation in the experimental plot 15 (covered with soil and sowed with *Dactylis glomerata*).

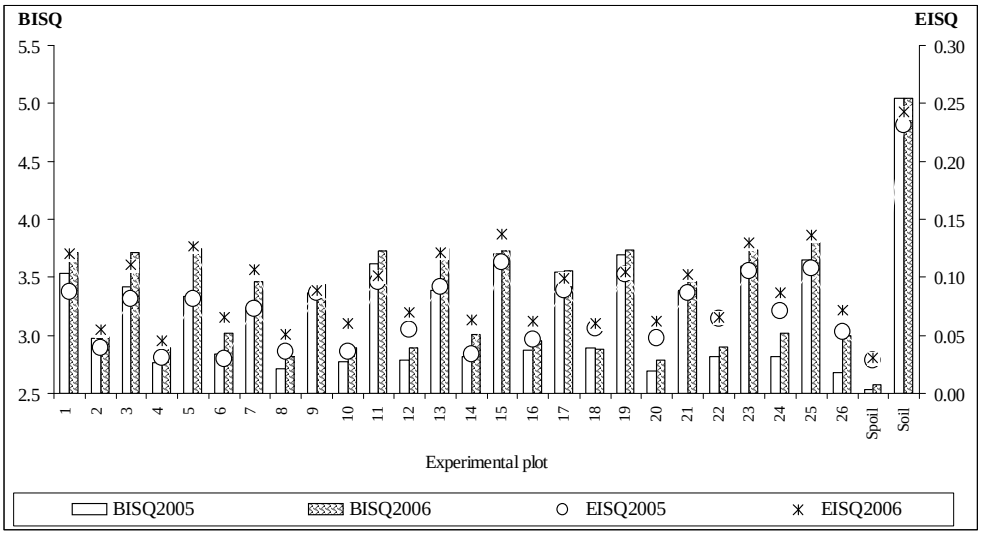

Fig. 1. Evolution of the bacterial (BISQ) and enzymatic (EISQ) indicators of soil quality in the experimental plots installed on the mine spoil dump in Iara.

It is also very obvious the zig-zag-like trajectory of both the BISQ, and the EISQ curves: the odd plots (covered with soil) have higher values of the indicators than the even ones (uncovered with soil). In the hierarchy of the experimental plots, based on the values of the two indicators, the odd plots are always situated on the first 13 positions, showing that the most important treatment applied to the experimental plots is the covering with a 10-cm layer of soil. The effect of the vegetal species used for sowing is not as important as the covering with soil is.

The most important remark from the analysis of the fig. 1 is the obvious increase of both bacterial and enzymatic potential in all the experimental plots. This increase certify the efficiency of the applied bioremediation technologies. The hierarchy of the experimental plots after one year from the beginning of the experiment, on the base of the BISQ and EISQ values is presented in tab. 2. Taking into account both the

bacterial, and the enzymatic indicators, we can assert that the best evolution of the general biological potential in the soils of the experimental plots after one year of vegetation was registered in the following plots, all of them covered with soil:

- plot 25: sowed with *Medicago sativa*; BISQ = 3.799 (the highest value); EISQ = 0.136 (the second value);
- plot 15: sowed with *Dactylis glomerata*; BISQ = 3.728 (the second value); EISQ = 0.137 (the highest value);
- plot 23: sowed with *Trifolium pratense*; BISQ = 3.733; EISQ = 0.130;
- plot 5: sowed with *Trifolium repens*; BISQ = 3.742; EISQ = 0.126;
- plot 13: sowed with *Festuca rubra* + *Trifolium pratense*; BISQ = 3.744; EISQ = 0.122.

Table 2.

Hierarchy of the experimental plot after one year from the beginning of the experiment, according to the BISQ and EISQ values

Pozition	Plot	BISQ	Plot	EISQ
1	25	3.799	15	0.137
2	13	3.744	25	0.136
3	5	3.742	23	0.130
4	19	3.741	5	0.126
5	23	3.733	13	0.122
6	15	3.728	1	0.120
7	11	3.722	3	0.111
8	1	3.716	7	0.106
9	3	3.712	19	0.105
10	17	3.557	21	0.102
11	7	3.459	11	0.101
12	21	3.457	17	0.099
13	9	3.438	9	0.089
14	6	3.019	24	0.087
15	24	3.015	26	0.071
16	14	3.002	12	0.070
17	26	3.001	22	0.066
18	2	2.987	6	0.065
19	16	2.954	14	0.064
20	22	2.898	20	0.063
21	4	2.891	16	0.062
22	10	2.889	18	0.060
23	12	2.886	10	0.060
24	18	2.880	2	0.055
25	8	2.814	8	0.051
26	20	2.789	4	0.045

From this point of view, we notice that after one year of vegetation, the most suited species to be used for bioremediation of such spoils proved to be *Medicago sativa*: the plot 25 (covered with soil) was situated on the first position

as regard the BISQ, and on the second one, as regard the EISQ; at the same time, the corresponding plot uncovered with soil – 26 – occupies the position 4 (BISQ), and 2 (EISQ), respectively, between the 13 experimental plots uncovered with soil.

We mention that, in parallel with the experimental plots analyzed in the present work, the bioremediation experiment was completed by planting on the slopes of the spoil dump saplings of several ligneous species: *Hippophaë rhamnoides, Robinia pseudacacia, Betula pendula, Ailanthus altissima* etc. Both the ligneous, and the herbaceous plants used for sowing the experimental plots analyzed here had a good evolution during the first two years of vegetation.

Conclusions

The microbiological analyses carried out three months after the initiation of the recultivation experiment and one year later registered the presence of the aerobic mesophilic heterotrophs, ammonifiers, denitrifiers and iron-reducers in all the experimental plots, and the lack of the sulphate reducing bacteria in all the plots uncovered with soil, both at the beginning of the experiment, and after one year. The sulphate reducing bacteria were present only in 9 experimental plots at the beginning, and in all the 13 experimental plots covered with soil, but their number was the lowest, as compared to the other bacterial groups.

The catalase and phosphatase activities were registered in all the experimental plots, at higher values in the plots covered with soil, than in the uncovered ones, both at the beginning of the experiment, and after one year of vegetation. No actual or potential dehydrogenase activity was detected in the plots uncovered with soil in the first year. After one year of vegetation, the potential dehydrogenase activity was registered in all the experimental plots.

The plots covered with soil have higher values of both bacterial, and enzymatic indicators of soil quality than the plots uncovered with soil, demonstrating that the most important treatment applied to the experimental plots is the covering with a 10-cm layer of soil. A strong positive correlation, statistically very significant ($p<0.001$) has been established between the bacterial and the enzymatic indicators of soil quality.

A remarkable increase of both bacterial, and enzymatic potential, defined by the BISQ and EISQ values, has been registered in all the experimental plots after the first year of experiment, certifying the efficiency of the applied bioremediation technologies.

The best evolution of the general biological potential in the soils of the experimental plots after one year of vegetation was registered in the plots covered with soil and sowed with *Medicago sativa*, *Dactylis glomerata*, *Trifolium pratense*,*Trifolium repens* and with a *Festuca rubra* + *Trifolium pratense* mixture.

On the base of the values of both the bacterial, and the enzymatic indicators of soil quality after one year of vegetation, one can be stated that the most suited species to be used for bioremediation of such spoils is *Medicago sativa*.

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MICROORGANISM POPULATIONS WITHIN GELATINOUS FORMATIONS FROM KIESBERG MINE IN BANAT MOUNTAINS, ROMANIA

VASILE-DANIEL GHERMAN[1], JEAN-GABRIEL BRÉHERET[2] AND MIHAIL-DRĂGAN BULARDA[3]

SUMMARY. This study concerns the gelatinous formations found in the Kiesberg gallery, part of an old mine located in the South-West of Romania which was dug in the metamorphic rocks of auriferous sulphides from the Policarpus ore. These gelatinous formations are being supplied with a variable flow of infiltration rainwater. Microbiological determinations and numerical estimates have been carried out in years 2004 and 2005, for two hydrological different seasons, a wet one and a dry one. A percentage estimate of different physiological groups has also been realized. The gelatinous formations from the Kiesberg mine represent a complex assemblage formed by (1) chemolitotroph acidophilic microorganisms (water pH 2.8) which dominate during stages of high water supply and (2) by heterotrophic microorganisms rather abundant during stages of low water supply. The ratio between the two kinds of microorganisms is therefore variable depending on the water supply and other environmental factors.

Keywords: *Acidithiobacillus*, Actinomycetes, auriferous sulphides, autotrophs, fungus, gelatinous formations, heterotrophs, old abandoned mine, Policarpus ore.

Introduction.

This study refers to an old mine, the Kiesberg Mine, located in the South-West of Romania, nearby the town of Oravita (Fig. 1). This mine was worked until the end of the XIXth century and it was dug in the auriferous sulphides metamorphic rocks of a Policarpus ore.

In this mine, the presence of some microbiological gelatinous formations was noticed. The microorganisms presence in the gelatinous formations is evidenced by the significant large biomass accumulations (the stalactites and the gelatinous deposits) and also by the particular morphological and structural speleothems.

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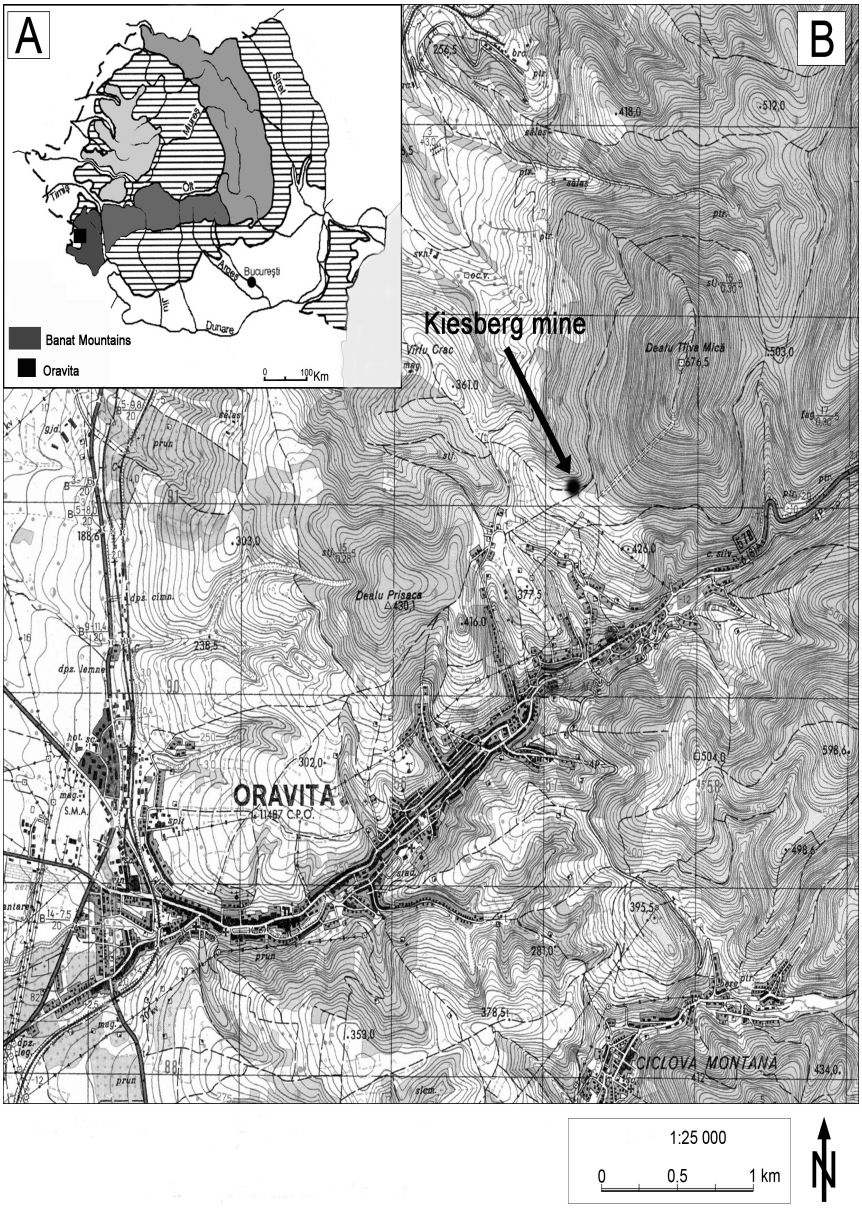

Fig. 1. Study area.

This type of gelatinous formations is not characteristic only for this kind of cavity, they are also often found in the ferous and sulphurous artificial (mines) or natural cavities (caves).

According to the specialized literature, the most well known and often found types of gelatinous formations are the ones from the hydrothermal cavities. These types of gelatinous formations are supplied with thermal and mezothermal waters with temperatures that vary between 18 and 50^0C. Sometimes, these waters come from active volcanic areas that generally contain great quantities of sulphur or sulphuretted hydrogen.

Here are some examples of thermal and mezothermal cavities where the gelatinous formations were found: the Lechuguilla Cave from the Carlsbad National Park, New Mexico, USA (Cunningham *et al*., 1995); the Cueva de Villa Luz from the Plateau Tabasco in Mexic (Northup *et al*., 1997); the Frassassi Cave from Italy (Vlăsceanu *et al.*, 2000); the Movile Cave from Dobrogea, Romania (Vlăsceanu *et al*., 1997).

In Wales, UK, there is an artificial cavity, a pyrite mine, closed more than 70 years ago – the "Cae Coch" Mine (Johnson, 1998; Johnson *et al.*, 1999; Hallberg *et al.*, 2006) – which is similar to Kiesberg Mine that we studied about.

The gelatinous formations of microorganisms' consortium can be differently formed, depending on several environmental factors: the cavity climate, the temperature of the water in which they are found, the chemical composition of the rock that forms the cavity and the chemical composition of the waters that drain various minerals (Hallberg *et al*., 2006).

The geological characteristics of the area where the Kiesberg mine is have induced the existence of a particular kind of medium and of microbial associations that form a series of characteristical gelatinous formations. The number of the microorganisms from these microbial associations varies quit a lot, depending on the rain water quantities during the year. In this study, the identification of characteristical gelatinous formations from the microbial associations in the Kiesberg mine is suggested.

The numerical estimations depending on the rainfall quantities (water that supllies the gelatinous formations) are also a part of this study.

Microbiological determinations and numerical estimates have been carried out in years 2004 and 2005, for two hydrological different seasons, a wet one and a dry one. Estimates of populations from different physiological groups have been calculated.

Materials and Methods.

The samples for the microbilogical analisys have been taken from a variable flow water stream found in a partially collapsed gallery from Kiesberg mine.

The isolation of acidophilic chemolitotrophic bacteria was performed by inoculating the growth medium (9K – pH 2.5) (Silverman *et al.*, 1959) for the isolation of *Acidithiobacillus ferrooxidans* (Kelly *et al*., 2000) *(Thiobacillus ferrooxidans)*, the Winogradski medium (W_6 –pH 6) (Winogradski, 1952) for other ferrous-bacteria, and the Starkey medium (S_6 – pH 6) (Starkey *et al*., 1934,) for *Acidithiobacillus thiooxidans (Thiobacillus thiooxidans)*.

The isolation of acidophilic heterotrophic microorganisms has been realized trough enriched growing on the March medium- pH-2.5 (Wood *et al.*, 1983), and on the G.Y.E. at pH 3 (Johnson *et al*., 1983).

The isolation and identification of fungi has been realized on the solid Czapeck medium (Ramirez, 1982), (Lorinczi *et al.*, 1966), starting from the serial dilutions of the enriched growing realized from samples in the liquid March medium.

The indirect determination of *Acidithiobacillus* strains by the measurement of pH medium was achieved by seeding the test samples on the 9K, S_5 and S_6, and also on the W_6 medium. The growing was determined by the quantity of oxidized thiosulphate. The *Acidithiobacillus* is estimated by the final pH value after 28 days (Hutchinson *et al*., 1966).

The determination of the most probable number of the microorganisms was done by performing decimal or serial dilution, with 3 repetitions/dilution, while the observation of the number of positive tubes, the numerical value, was established from the table (Rodina, 1972). The method of counting the colonies on the solid medium was used also for the numerical estimates.

Results and Discussion

In 2004, the measurements were realized in March, when the total rainfall value was 29,9 mm (the dry season) and in July , when the total rainfall value was 141,8 mm (the rainy season). In 2005, the measurements were realized in November, when the total rainfall value was 37,4 mm (the dry season) and in April, when the total rainfall value was 141,8 mm (the rainy season). (From a hydrological point of view) 2004 was a drier year (875 mm) than 2005 (1202 mm).

The results of the microbiological analysis made on the gelatinous formations from Kiesberg mine are given in Table 1.

The fact that 16 strains of microorganisms were identified in 2004 can be noticed from Table 1. For the next year, 2005, one more strain – *Scytalidium (Dematiaceae Family)* – was identified beside the 16 ones.

The identified microorganisms strains are intimately associated by extra-cellular mucopolysaccarides, forming an association or a consortium. From all the identified strains, the four *Acidithiobacillus* together with the actinomycetes represent the bacteria, the rest of the strains represent the fungi. *A. neapolitanus, A. thiooxidans* and *A. thioparus* represent acidophilic chemolitotrophic bacteria which realize the oxidation of the reduced sulphur compounds. These compounds stem from the chemical and biological oxidation of pyrite from the Kiesberg gallery walls. In the consortium, the *Acidihiobacillus* represent the primary organic substance producers. *A. intermedius* is a strain with a mixotrophic metabolism; it is a facultative heterotroph.

Table 1.

Microorganisms identified in the gelatinous formations in Kiesberg Mine and their most probable number in the two seasons during the years of 2004 and 2005**.**

Strain	2004		2005	
	rainy	dry	rainy	dry
Acidithiobacillus neapolitanus	9500	0	9500	1400
Acidithiobacillus thiooxidans	9500	3000	9500	4500
Acidithiobacillus thioparus	4500	0	7500	2000
Acidithiobacillus intermedius	4500	3000	4500	4500
Actinomicete	45000	4500	9500	4500
Penicillium citreo-viride	200	0	300	200
Penicillium implicatum	600	300	700	0
Penicillium expansum	400	200	600	200
Penicillium veruncosum	300	0	200	0
Penicillium veruncosum var. corimbiferum	2000	200	300	0
Penicillium griseo-fulvus	400	0	700	300
Penicillium steckii	200	0	200	0
Aspergillus nidulans	400	0	600	200
Aspergillus thomii	600	300	700	300
Rhodotorula sp.	400	300	600	400
Cladosporium herbarum	0	300	0	200
Scytalidium sp.	0	0	200	0

The autochthonous organic substance, from the primary producers (the *Acidothiobacillus*), together with allochthonous organic substances stemming with the water from other parts of the cavity, is scavenged by the heterotrophic microorganisms from this consortium. The heterotrophs are represented by actinomycetes, whose strains, unfortunately, haven't been identified yet, and by the following strains of fungi (Lorinczi *et al.*, 1966): Fam. *Moniliaceae: Aspergillus nidulans, Aspergillus thomii, Penicillium steckii, P. expansum, P. verucosum, P. griseo-fulvus, P. implicatum, P. verucosum var. corybiferum ;* Fam. *Cryptococaceae: Rhodotorula sp.;* Fam. *Dematiaceae: Cladosporium herbarum.* Unidentified Protists in the gelatinous formations were also noticed by microscopy.

The bacterial strains are largely dominating, with 90 % of the population, the fungus strains representing only 10 %.

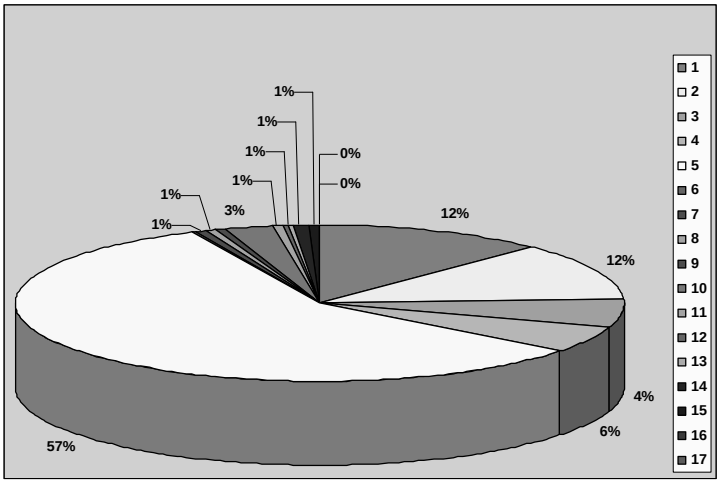

Fig. 2. The percentage of identified microorganisms strains during the rainy season 2004 in the gelatinous formations.

1. Acidithiobacillus neapolitanus; 2. A. thiooxidans; 3. A. thioparus; 4. A. intermedius; 5. Actinomicete; 6. Penicillium citreo-viride; 7. P. implicatum; 8. P. expansum; 9. P. veruncosum; 10. P. veruncosum var. corimbiferum; 11. P.griseo-fulvus; 12. P. steckii; 13.Aspergillus nidulans; 14. A. thomii; 15. Rhodotorula sp.; 16. Cladosporium herbarum; 17. Scytalidium sp.

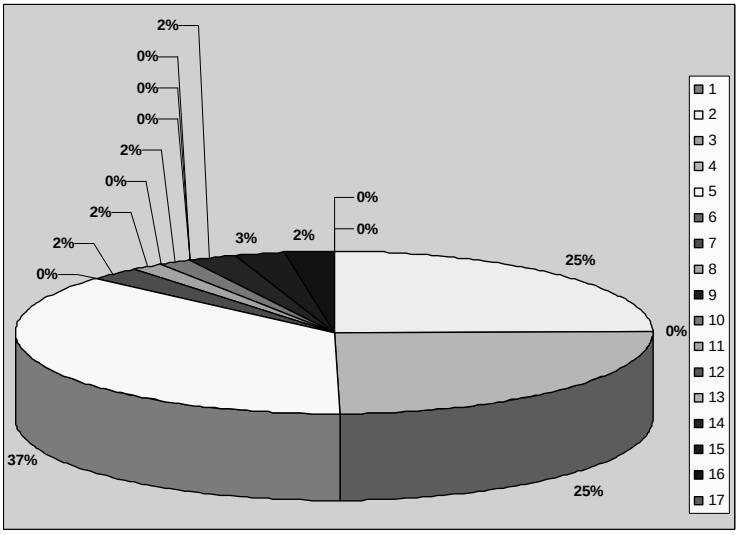

Fig. 3. The percentage of identified microorganisms strains during the dry season 2004 in the gelatinous formations.

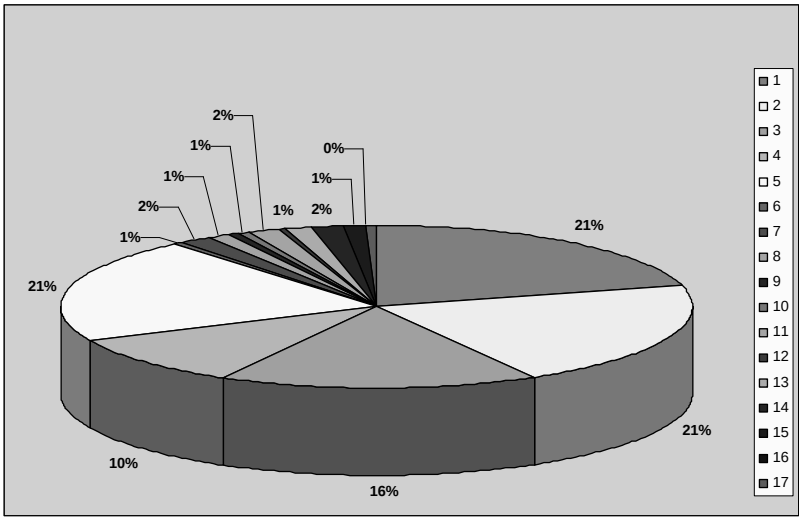

Fig. 4. The percentage of identified microorganisms strains during the rainy season 2005 in the gelatinous formations.

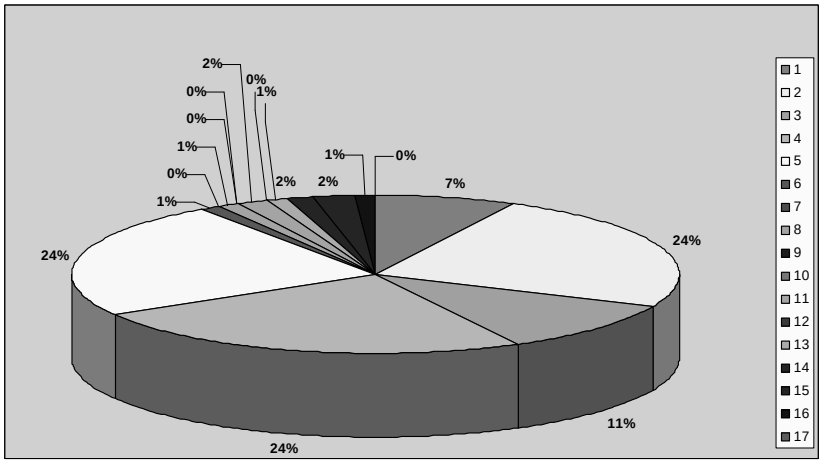

Fig. 5. The percentage of identified microorganisms strains during the dry season 2005 in the gelatinous formations.

1. Acidithiobacillus neapolitanus; 2. A. thiooxidans; 3. A. thioparus; 4. A. intermedius; 5. Actinomicete; 6. Penicillium citreo-viride; 7. P. implicatum; 8. P. expansum; 9. P. veruncosum; 10. P. veruncosum var. corimbiferum; 11. P.griseo-fulvus; 12. P. steckii; 13.Aspergillus nidulans; 14. A. thomii; 15. Rhodotorula sp.; 16. Cladosporium herbarum; 17. Scytalidium sp.

In the rainy season 2004 the heterotroph populations were dominating, the actinomycetes and fungi represented 66 %, while the chemolitotrophs represented 34

%. During the dry season 2004, the chemolitotrophs population (50 %) represented as much as the heterotrophs did (50%), with the observation that *A. intermedius* is part of facultative heterotrophs.

Table 2.
The physiological groups' ratios of microorganisms from the gelatinous formations, during the wet and dry seasons during 2004 and 2005**.**

year	season	autotrophs	heterotrophs
2004	**Rainy**	34%	66%
	Dry	50%	50%
2005	**Rainy**	68%	32%
	Dry	66%	34%

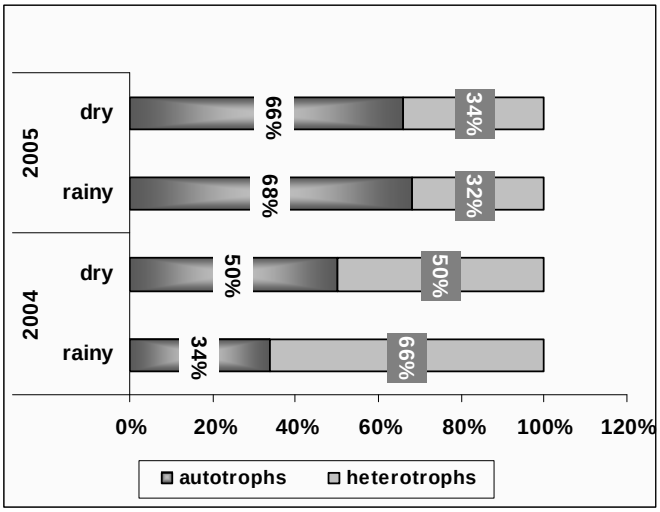

Fig. 6. The ratio of physiological groups of microorganisms from the gelatinous formations, during the rainy and dry seasons of 2004 and 2005.

During the rainy season 2005, the dominant strains were the chemolitotrophic *Acidithiobacillus* (68 %) among which *A. intermedius* is 10 %. The heterotrophs represented 32% with 21 % actinomycetes, and 11% of fungus strains (of the total population). During the dry season 2005, as well as during the rainy one, the chemolitotrophic *Acidithiobacillus* strains were dominant (66 %), among which *A. intermedius* (24 %). The fungus strains represent 10% of the total population (table 2. Fig. 6.).

The results of chemical analysis for the water that contains this consortium show an acid pH (2.81) and large quantities of sulphate (2810,5 mg/l). These results prove the existence of an intense oxidation of all reduced sulphate forms that are reduced by the chemolitotrophic bacteria. In 2004 and 2005, we can observe a lower variation of the microorganisms populations, from the rainy season to the dry one, during 2005 as compared with 2004. During 2004 the actinomycetes underwent a very high variation from the rainy season to the dry one however this fact is probably a random and short term situation.

The primary producers, represented by the *Acidithiobaccilus* strains, oxidize pyrite and other metallic sulphides from Kiesberg mine walls, but they also induce the oxidation of Fe^{2+} and reduced sulphur compounds which result from the chemical and biological pyrite oxidation. For example, the biological oxidation of pyrite (which is effective at the contact spot of gelatinous formations with the gallery walls) is very intense during the period of high water supply, because this water takes over the oxidation products. The local accumulations of oxidized products are inhibitors for *Acidithiobaccilus*. The products generated by the pyrite oxidation, during a first stage, are quickly oxidized by the chemolitotroph consortium that are found at a certain distance from the rock. So it can be explained that during the more rainy year of 2005 (1202.2 mm) autotrophic microorganisms (chemolitotrophs) prevailed, while the heterotrophs were rather abundant during the dryer year 2004 (875 mm).

In the Cae Coch Mine from Wales, the same as in the mine that we studied, the development of gelatinous microbial communities is based on the metalic sulphides oxidation from the rock mass (Johnson, 1999). The main chemo-synthesized autotrophs strains from this mine community belong to the *Acidithiobacillus* type and the specific hetrotrophs are the heterotrophic eubacteria, the actinomycetes, a series of eukaryotes (like Deuteromycetes and Protists strains) and, very rarely, micro-unvertebrated (such as Nematods) (Hallberg *et al.*, 2006).

From the biodiversity point of view, the gelatinous formations from the Kiesberg Mine that we studied, and also the ones from the Cae Coch Mine, are less rich in strains compared to the gelatinous formations from the hydrothermal cavities. Even so, the biomass formed by microorganisms in the mezothermal mines is quite large. In our case, the gelatinous formations biovolume is a few cubic meters, but in the Cae Coch Mine the biovolume is beyond $100m^3$ (Johnson, 1999; Hallberg *et al.*, 2006).

Conclusions

The gelatinous formations represent a complex association formed by heterotrophic, autotrophic (chemolitotrophs) and acidophilic microorganisms, which interact very closely. The ratio between them varies, mainly depending on the water supply.

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STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LII, 2, 2007 (p.119-127)

MICROORGANISM ASSOCIATIONS WITHIN A THIN ACID SOLUTION FILM FROM AN OLD MINE IN BANAT MOUNTAINS

VASILE-DANIEL GHERMAN [1], JEAN-GABRIEL BRÉHERET[2] AND MIHAIL-DRĂGAN BULARDA[3]

SUMMARY. The rain water slowly crosses the rock layers down to the cavity through micro cracks and it arrives on the cavity wall as a concentrated solution with a high acidity level (pH 1-1.5). This concentrated acid solution was subjected to some microbiological determinations and the identified strains were numerical estimated. These determinations were done in 2004 and 2005, during two hydrological different seasons, rainy and dry. A percentage estimate of the physiological groups has also been done.

The percolating acid solution is crowded by acidophilic microorganisms with a dominating population of *Acidithiobacillus ferrooxidans* and six strains of *Penicillium*. The chemolitotrophic bacteria *A. ferrooxidans* provides the primary organic substance production of the community by oxidation of pyrite from the cavity walls. The six strains of *Penicillium* represent the heterotrophic organisms that use the organic substance from the primary producer. Between the *A. ferrooxidans* population and the *Penicillium* strain populations a close trophic relationship is established and the microorganisms number depends on the acid solution concentration.

Keywords: acid solution, auriferous sulphides, autrotrophs, heterotrophs, old mine, *Penicillium*, *Acidithiobacillus ferrooxidans*.

Introduction

Highly acid medium limit the access of the organisms that are unable to adapt to this stress, and are considered as "extreme life medium" (Johnson, 1998).

The studies on the acid medium have revealed the great diversity of organisms that are part of three sistematic fields: Eubacteria, Archaea and Eucaria. Between the acidophilic organisms populations there are many interactions (connections), especially trophic kind connections.

The most numerous microbiological studies have been done on the mine drainage waters: in Iron Mountain, California (Schrenk *et al.*, 1998; Edwards *et al.*, 2000)**;** in Scandinavia and the United Kingdom (Banks *et al.*, 1997); Alaskan and Canadian drainages gold mines (Braddock *et al.*, 1984); in Norwegian copper mine (Johnson *et al.*, 2001); these waters represent an important polution source.

This study took place in an old mine (the Kiesberg Mine), abandoned at the end of the XIXth century, located in the South-West of Romania, nearly Oravita town (Fig. 1.).

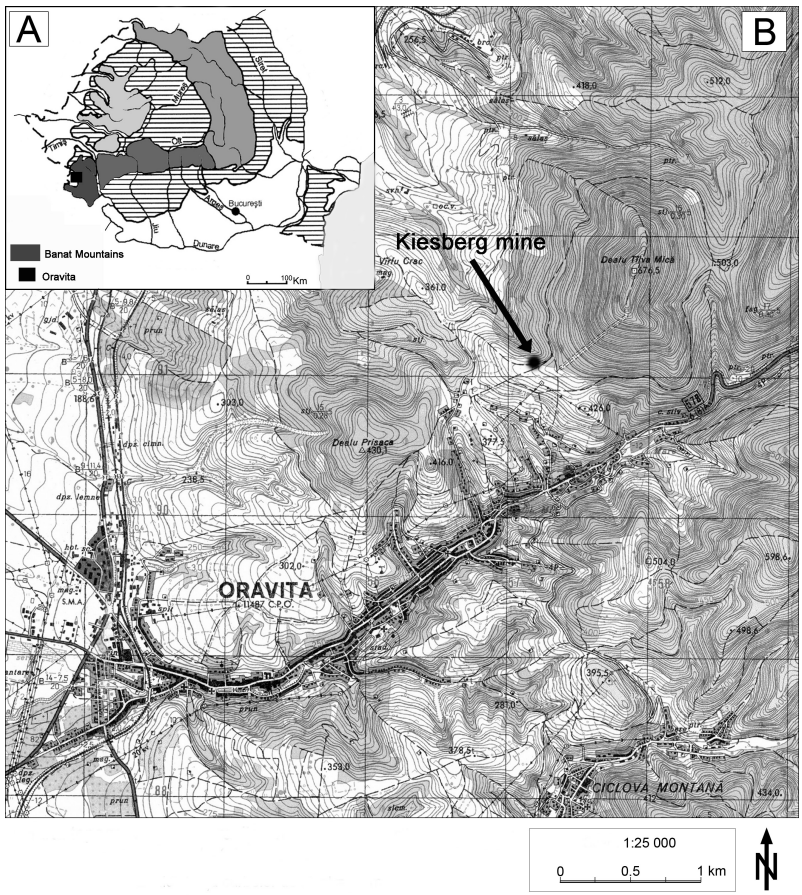

Fig. 1. Study area.

This mine is located in the Banat Mountaine and it was dug in the metamorphic rocks of auriferous sulphides of Policarpus ore. The thickness of the metamorphic rocks above the cavity is at least 8 meters; they are covered by several meters of limestones and marls. The rainwater crosses the overburden through micro-cracks and rises on the cavity wall. The flowing period through micro cracks is of 2 weeks. During this period, the rainwater dissolves a series of minerals: silicates and iron sulfides. As it springs up on the vault of the cavity it is a highly acid solution (pH 1-1,5). Various kinds of speleothems are growing in this context (the snotites and stalactites formations). As organic matter is

intimately linked to mineral production in these stalactites, so arise the question of the contribution of organisms (1) to the leaching of the minerals from the source rock and (2) to the new mineral deposits.

The extremely particular medium from the Kiesberg cavity induces the presence of characteristical microorganisms associations that are physiologically and ecologically very interesting. In this study, the identification, in two different hydrological seasons, a wet one and a dry one, during the years of 2004 and 2005, of characteristical microorganisms associations which populate the walls of this old mine, was suggested proposed. A percentage estimation of different physiological groups is presented further on.

Materials and Methods

These samples have been taken from a continous, permanent acid solution film which covers the vault of one part from the profound area of Kiesberg mine.

The isolation of acidophilic chemolitotrophic bacteria was performed by inoculating the growth medium (9K – pH 2.5) (Silverman *et al.*, 1959) for the isolation of *Acidithiobacillus ferrooxidans* (Kelly *et al*., 2000) *(Thiobacillus ferrooxidans)*, the Winogradski medium (W_6 –pH 6) (Winogradski, 1952) for other ferrous-bacteria, and the Starkey medium (S_6 – pH 6) (Starkey *et al*., 1934,) for *Acidithiobacillus thiooxidans (Thiobacillus thiooxidans).*

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The isolation and identification of fungi has been realized on the solid Czapeck medium (Ramirez, 1982), (Lorinczi *et al.*, 1966), starting from the serial dilutions of the enriched growing realized from samples in the liquid March medium.

The indirect determination of *Acidithiobacillus* strains by the measurement of pH medium was achieved by seeding the test samples on the 9K, S_5 and S_6, and also on the W_6 medium. The growing was determined by the quantity of oxidized thiosulphate. The *Acidithiobacillus* is estimated by the final pH value after 28 days (Hutchinson *et al*., 1966).

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Results and discussion

In 2004, the measurements were realized in March, when the total rainfall value was 29,9 mm (the dry season) and in July , when the total rainfall was 141,8 mm

(the rainy season). In 2005, the measurements were realized in November, when the total rainfall value was 37,4 mm (the dry season) and in April, when the total rainfall was 226,4 mm (the rainy season). (From a hydrological point of view) The year 2004 was drier (875 mm) than 2005 (1202 mm).

The results of the microbiological analysis performed on this acid solution are presented in Table 1.

Table 1.

Microorganisms identified in the acid solution and their most probable number during the two seasons of 2004 and 2005

Strain	2004		2005	
	wet	dry	wet	dry
Acidithiobacillus ferrooxidans	9500	4500	9500	8000
Penicillium expansum	600	500	600	600
Penicillium cyclopium	200	150	250	150
Penicillium frequentans	300	200	500	300
Penicillium griseo-azureum	0	200	0	200
Penicillium citreo-viride	200	0	300	200
Penicillium lividum	200	200	300	300

As it is indicated on the table 1, seven strains of microorganisms forming a consortium, and thus functionally interconnected, were determined in the acid solution, both in 2004 and 2005. *Acidithiobacillus ferrooxidans (Thiobacillus ferrooxidans)* is the only bacterial strain, the rest being represented by six strains of *Penicillium* fungi, which are a part of *Moniliacea Family*. Their percentages are depicted on the Fig. 2-5.

In 2004 and 2005, a clear domination of *Acidithiobacillus ferrooxidans* (80 %) is observed during both seasons; the remaining 20 % correspond to the *Penicillium* strains. *A. ferrooxidans* is a chemolitotrophic bacteria that oxidizes the pyrite, the Fe^{2+} and the sulphur compounds (stemming from the oxidation of pyrite), providing the primary production of organic substance; the six strains of *Penicillium* represent the heterotrophs that use the organic substance produced by *A. ferrooxidans.* (Table 2., Fig. 6.).

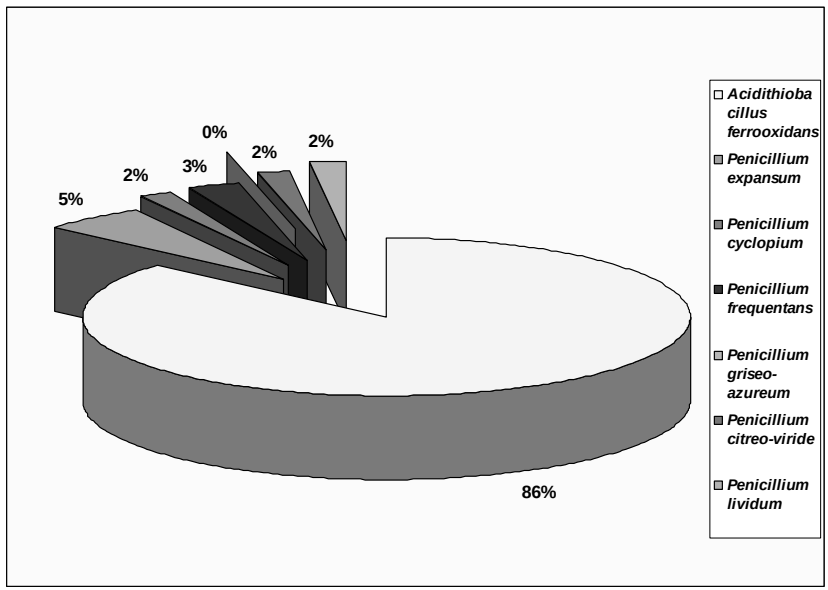

Fig. 2. The percentage of identified microorganisms strains during the rainy season 2004 in the acid solution.

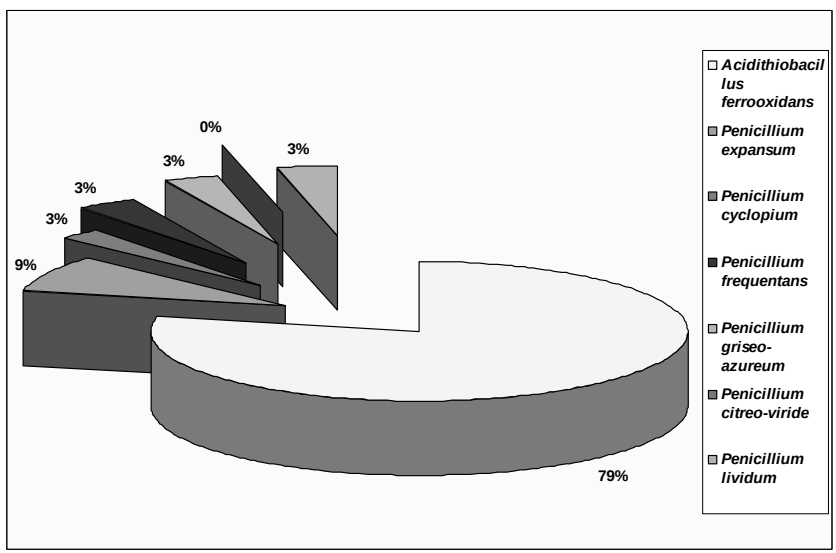

Fig. 3. The percentage of identified microorganisms strains during the dry season 2004 in the acid solution.

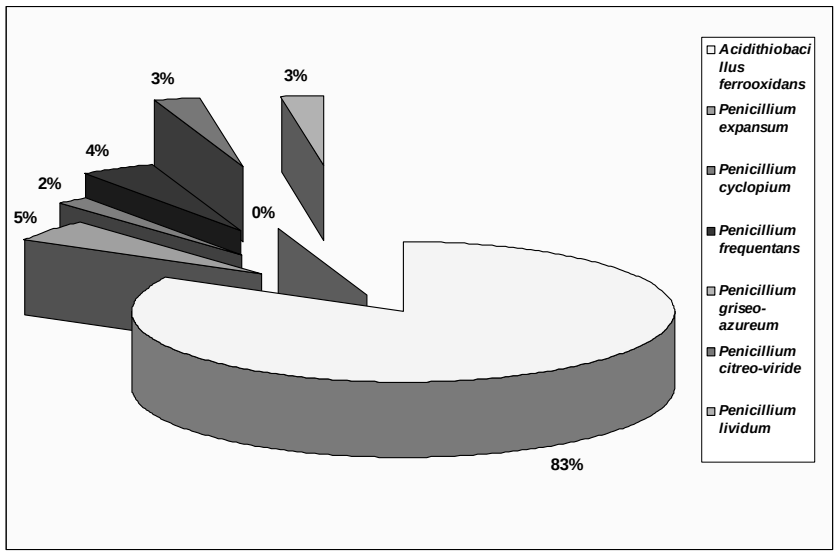

Fig. 4. The percentage of identified microorganisms strains during the rainy season 2005 in the acid solution.

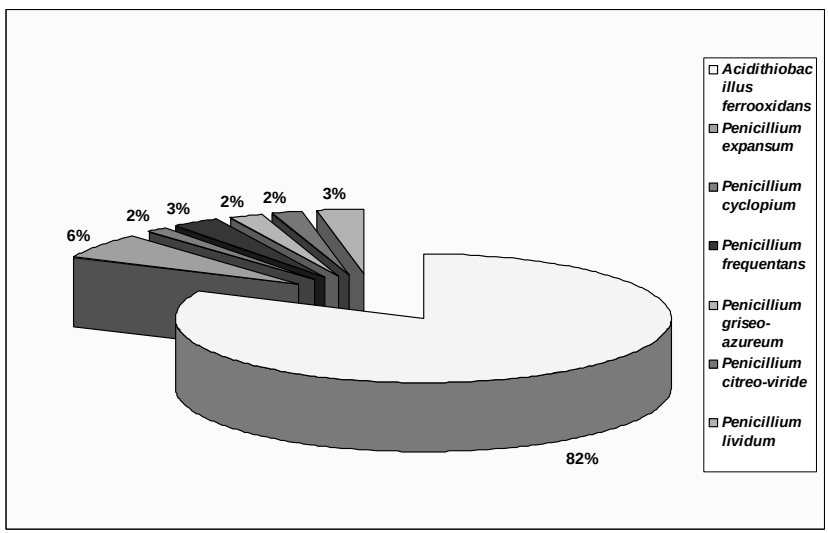

Fig. 5. The percentage of identified microorganisms strains during the dry season 2005 in the acid solution.

Table 2.
The ratio of microorganisms physiological groups from the acid solution, during the wet and dry seasons of 2004 and 2005.

year	season	autotrophs	heterotrophs
2004	**rainy**	86%	14%
	dry	79%	21%
2005	**rainy**	83%	17%
	dry	82%	18%

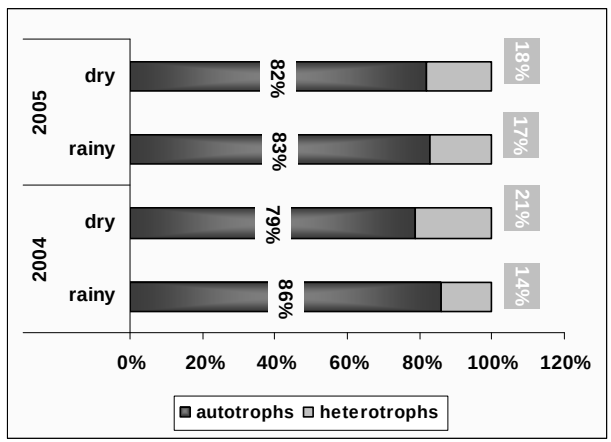

Fig. 6. The ratio of physiological groups of microorganisms from the acid solution, during the rainy and dry seasons of 2004 and 2005.

Acidithiobacillus ferrooxidans is usually isolated strain, known since 1950 as a metalic sulpide oxidizer (Colmer *et al.*, 1950; Temple *et al.*, 1951). This strain was found in the mine water drainage, with high acidity. The *Penicillium* types of fungi are heterotrophs eukaryotes that are often found on the organic substances from the acid waters (Ramirez, 1982; Baker *et al.*, 2004; López-Archilla *et al.*, 2004).

In the acid solution studied by us at the Kiesberg Mine the association of the *Acidithiobacillus ferrooxidans* with the six strains of *Penicillium* into a continuous biofilm on the cavity vault is characteristic to this mine. Close trophic type relationships are established between the organisms of this consortium.

Conclusions

The acid solution (pH 1.21) percolating in the Kiesberg mine overburden, houses a rich acidophilic community of microorganisms. This community is formed (1) by a numerous population of *Acidithiobacillus ferrooxidans* (80 %), that represents the primary producer of organic substance which oxidizes the pyrite from the cavity walls and (2) by the populations of six strains of *Penicillium* (20 %); these heterotrophic organisms use the organic substances provided by the primary producer.

The biological processes that take place in this acid solution from the cavity vault are continuous; they occur during the whole year, the acid solution layer being permanently supplied throughout the fine crack system from the cavity wall.

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MICROBIAL COMMUNITIES AND ENZYMATIC ACTIVITIES IN THE MOUNTAINOUS SOILS IN PARÂNG

RAHELA CARPA[1]

SUMMARY. Fifteen soil samples from the Parâng Mountain in the South-Eastern part of the Hunedoara county, from five altitudinal vegetation zones (alpine, subalpine, conifers, beech and flood plain) have been analyzed microbiologically and enzymologically. The microbiological analyses consisted in the study of the abundance, dynamics, diversity and ecological significance of the bacterial groups involved in the biogeochemical cycles of carbon and nitrogen: aerobic mesophilic heterotrophs, ammonifiers, denitrifiers, nitrate and nitrite bacteria. Based on the obtained results, the bacterial indicator of the soil quality (BISQ) was calculated for each type of soil in each altitudinal vegetation zones. In order to have a complete image on the complex microbial processes in these habitats, these analyses have been completed with enzymological researches which make possible the appreciation of evolution of the soil types. The phosphatase, catalase, actual and potential dehydrogenases activities have been studied in all the soil samples. The studied activities were detected in all the 5 altitudinal vegetation zones, with differences noticed only as regard the intensity of the processes. Based on the absolute values of the enzymatic activities, the enzymatic indicator of soil quality (EISQ) was calculated. The EISQ values ranged between 0.314 and 0.430, indicating a moderate intensity of the enzymatic activities. A positive correlation between the bacterial and enzymatic indicators of soil quality (r = + 0.976) was established.

Keywords: soil, bacterial communities, enzymatic activities, bacterial and enzymatic indicators of soil quality

Introduction

The importance of the microbiological and enzymological study of soils has been frequently underlined by researchers in this field (Zborovschi *et al.*, 1989; Paşca *et al.*, 1993; Drăgan-Bularda *et al.*, 1995). The two kinds of analyses are complementary and offer a general overview on the complex relations between the components of the soil microbiota, as well as on the enzymatic transformations of organic compounds in soils. The present paper aims to establish such relations in soils sampled from five altitudinal vegatation zones in the Parâng Mountain: the alpine zone, the subalpine zone, the conifers zone, beech zone and the flood plain from Maleia river. The microbiological and enzymological analyses pursue the knowledge of the percentage of some bacterial physiological groups and of some enzymes involved in the biogeochemical cycles of the elements and the achievement of an overall image of the biological activity in the soil.

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Material and Methods

Fifteen soil samples from the Parâng Mountain, as three samples from five altitudinal vegetation zone (tab. 2), have been analyzed microbiologically and enzymologically. The distance between the sampling points of a altitudinal vegetation zone was 250 m and the depth of sampling was 15 cm.

There was established the number of bacteria belonging to the following ecophysiological groups: aerobic mesophilic heterotrophs, ammonifiers, denitrifiers, nitrate bacteria and nitrite bacteria. All operations connected to the bacteriological determinations were carried out under sterile conditions. There was also established the content of the soil in dry substance, by the drying of some samples at 105^{o}C, for three days. The number of the aerobic mesophilic heterotrophs was determined on a plates bullion agarized medium (Atlas, 2004). After incubation the number of colonies in each Petri dish was read, the average of the parallel sample 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 nitrite – reducing bacteria a selective liquid medium (Drăgan-Bularda, 2000) was used. The most probable number of the bacteria was calculated according to the statistical table of Alexander (1965).

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

Results and Discussions

The results of the microbiological analyses are presented in tab. 1. One can notice the presence of all the five ecophysiological groups in all the analyzed samples.

It is remarkable the large number of the aerobic mesophilic heterotrophs of order 10^6 - 10^7 cells/g soil of dry substance, in the soil of the alpine altitudinal vegetation zone and in the samples of the Maleia flood plain.

The number of bacteria belonging to the other groups is much smaller. In the order of their abundance, the aerobic mesophilic heterotrophs are followed by the ammonifiers (10^3 – 10^5 cells/g soil dry substance), denitrifiers (10^3 – 10^4 cells/g soil dry substance), nitrate-reducing (10^3 – 10^4 cells/g soil dry substance), the weakest represented are the nitrite-reducing bacteria (10^3 cells/g soil dry substance).

Table 1.

The results of the microbiological analyses

Soil type	Number of the bacteria/ g soil of dry substance					BISQ
	Aerobic mesophilic heterotrophs	Ammonifiers	Denitrifiers	Nitrate bacteria	Nitrite bacteria	
Alpine	25184772	45287	8444	8096	1925	4.635
Subalpine	18337853	40520	7555	8376	1992	4.594
Conifers	7339027	1350	1243	4739	1127	3.964
Beech	17619239	42675	15378	6740	2164	4.645
Flood plain	30162846	123164	17447	10231	1802	4.815

As in the case of the aerobic mesophilic heterotrophs, in the soil from the alpine and flood plain samples the number of the ammonifiers, denitrifiers, nitrate and nitrite-reducing bacteria is larger than the number registered in the other three sample soils.

On the base of the number of bacteria belonging to each ecophysiological group, the bacterial indicators of the soil quality (BISQ) were calculated, according to Muntean, (1995-1996):

$$IBCS = \frac{1}{n} \bullet \sum \log_{10} N$$

where BISQ = the bacterial indicator of the soil quality;
n = the number of the physiological groups considered within the calculation;
N = the number of the bacteria belonging to each ecophyisiological group.

As we can notice in fig. 1, the microbial potential of soils, defined by the values of the bacterial indicators of soil quality is different. Thus, the soil in the alpine altitudinal vegetation zone and in the flood plain has the highest bacterial potential and the soil in the conifers altitudinal vegetation zone has the lowest potential. The differences are not very high. Only the soil in the conifers zone has lower value of the BISQ (3.964).

The results of the enzymological analyses are presented in tab. 2. In all the samples analyzed there was remarked the presence of each of the four studied enzymes, the intensity of the activities varies within larger limits, in the case of the phosphatase and within restricted limits in the case of catalase.

As compared with the data in the literature (Zborovschi *et al.*, 1989; Drăgan-Bularda, *et al.*, 1995), we may consider that the phosphatase and especially catalase activities are intense, while the dehydrogenase activities have lower values.

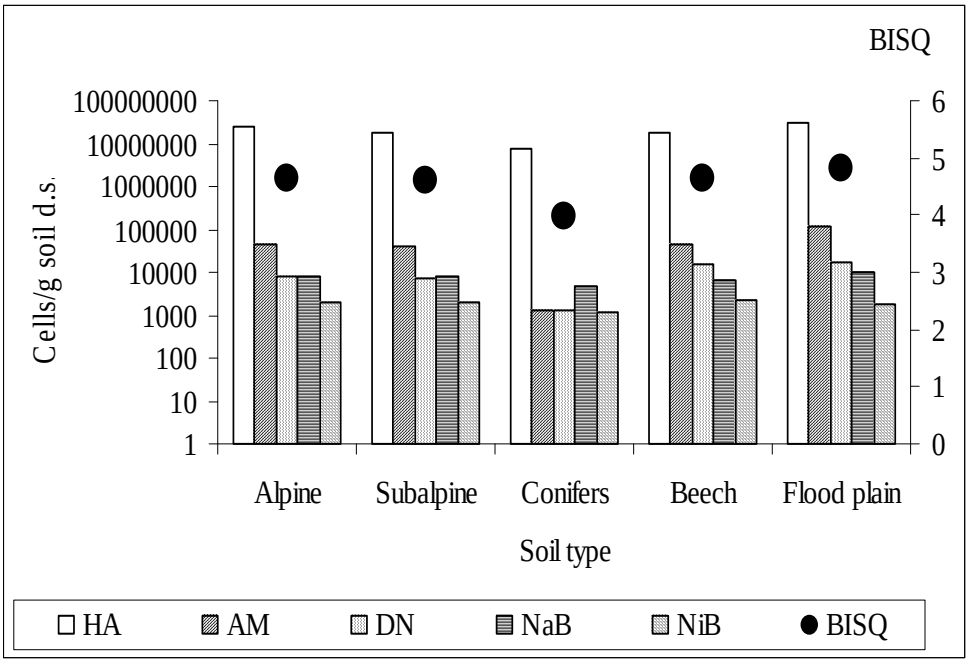

Fig. 1. Bacterial potential of the soil based on the values of the bacterial indicators of the soil quality (BISQ).

HA = aerobic mesophilic heterotrophs; AM = ammonifiers; DN = denitrifiers; NaB = nitrate bacteria; NiB = nitrite bacteria.

One might notice that the maximum values of each activity were achieved in the soil from the Maleia flood plain. The minimum values for the dehydrogenase activity were registered in the soil of the conifers zone, only the catalase activity registers the minimum value in the soil from the beech altitudinal vegetation zone.

Based on the absolute values of each enzymatic studied activity, the enzymatic indicator of the soil quality (EISQ) was calculated, according to the formula (Muntean *et al.*, 1996):

$$IECS = \frac{1}{n} \bullet \sum_{i=1}^{n} \frac{V_r(i)}{V_{max}(i)}$$

where EISQ = the enzymatic indicator of the soil quality;
n = number of activities;
$V_r(i)$ = real individual value;
$V_{max}(i)$ = maximum theoretical individual value.

The maximum theoretical individual value, calculated from the composition of the reaction mixtures are: 21.56 mg phenol (phosphatase activity), 60 mg splitted H_2O_2 (the catalase activity), and 13.45 mg formazan (dehydrogenase activities).

Table 2.

The results of the enzymological analyses

Soil type		Phosphatase activity (mg phenol/2,5 g)	Catalase activity (mg splitted H_2O_2 /1,5 g)	Dehydrogenase activity		EISQ
				Actual	Potential	
				mg formazan/2,5 g		
Alpine	a	13.346	45.823	1.579	2.970	0.430
	b	11.588	43.845	1.392	2.596	0.391
	c	10.060	45.164	1.889	2.868	0.393
Mean value		11.664	44.944	1.620	2.811	0.405
Subalpine	a	20.094	32.637	2.032	3.375	0.469
	b	15.379	34.285	2.350	2.149	0.405
	c	5.258	37.582	0.738	1.642	0.262
Mean value		13.577	34.834	1.707	2.389	0.379
Conifers	a	9.418	44.175	1.509	1.749	0.354
	b	9.693	32.637	0.581	0.726	0.273
	c	10.594	33.296	1.122	1.772	0.315
Mean value		9.902	36.703	1.071	1.416	0.314
Beech	a	17.343	30.659	1.364	2.120	0.394
	b	11.331	27.032	1.181	0.843	0.282
	c	17.914	37.252	2.350	5.401	0.507
Mean value		15.530	31.648	1.632	2.788	0.394
Flood plain	a	16.764	26.703	0.967	1.738	0.356
	b	19.262	27.362	1.798	2.919	0.425
	c	15.501	47.472	3.450	3.639	0.509
Mean value		17.176	33.846	2.072	2.765	0.430

We mention that the enzymatic indicator may have values ranging between 0 (when no real activity of any of the studied enzymes is detected) and 1 (when all the activities have real individual values equal to the maximum theoretic values).

As in the case of the bacterial potential, the enzymatic potential of soils, defined by the values of the quality enzymatic indicators is represented in fig. 2. Only the flood plain soil exceeds the 0.5 value of the EISQ, and the value of soil from the conifers altitudinal vegetation zone is lower than 0.4 of the EISQ.

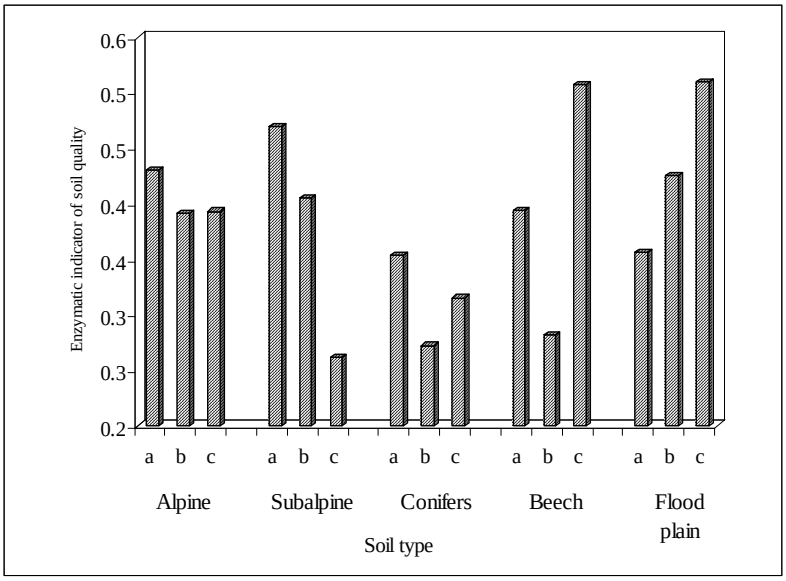

Fig. 2. The enzymatic potential of soils.

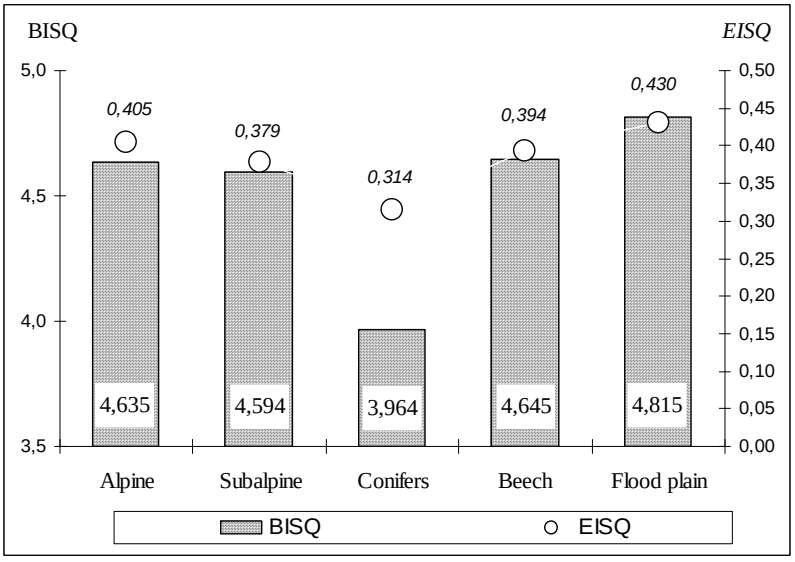

Fig. 3. The comparison of the microbial and enzymatic potential, on the base of the values of the bacterial (BISQ) and enzymatic (EISQ) indicators of soil quality.

Fig. 3 illustrates the bacterial and enzymatic potential of the analyzed soils, based on the quality indicators values. One can observe their parallel route, reflected, in fact by the positive correlation coefficient calculated between the two indicators (r = +0,976).

The bacterial and enzymatic indicators of the analyzed habitats quality offers an overall image on the intensity of the microbial activity and, implicitly, enzymatic, of the general biological activity in the analyzed soils. Based on the results and in comparison with the data in the specialty literature (Paşca *et al*., 1993; Drăgan-Bularda, *et al.*, 1995), we may consider that the analyzed soils have an appreciable biological potential; only the soil in the conifers altitudinal vegetation zone have lower values of the two quality indicators, bacterial and enzymatic, which set the basis for this appreciation.

Conclusions

The presence of all the five studied bacterial ecophysiological groups studied was noticed in all the soil samples. Their number decreases in the following order: aerobic mesophilic heterotrophs (10^6 - 10^7 cells /g soil dry substance) > ammonifiers (10^3 – 10^5 cells/g soil dry substance) > denitrifiers (10^3 – 10^4 cells/g soil dry substance) ≥ nitrate-reducing bacteria (10^3 – 10^4 cells/g soil dry substance) > nitrite-reducing bacteria (10^3 cells/g soil dry substance).

The microbial potential of soils, defined by the values of the bacterial indicators of soil quality decreases in the following order: flood plain soil > soil from the alpine altitudinal vegetation zone > soil from the subalpine altitudinal vegetation zone > soil from the altitudinal beech zone > soil from the conifers altitudinal vegetation zone. The differences among them are not significant. Only the soil in the conifer forests has a value of the bacterial indicator of quality smaller than four (3.964).

The presence of each of the 4 studied enzymes was detected in all the soils analyzed. The soil enzymatic potential, defined by the values of the enzymatic indicators of quality, decreases in the same order as the microbial potential. A positive correlation, with good statistical significance ($p<0.01$), was established between the bacterial and the enzymatic indicators of soil quality.

The flood plain soil proved to be a good soil from the enzymological and bacterial point of view. Both the enzymatic and bacterial indicators of soil quality reached the highest values: BISQ = 4.815 and EISQ = 0.430. This values indicate the presence of high enzymatic and bacterial potential in these zones as compared with the other analyzed zones.

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