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OVERLOOKED TRADITIONAL ORCHARDS: THEIR IMPORTANCE FOR BREEDING BIRDS

MILAN VOGRIN[1,✉]

SUMMARY. Traditionally used orchards are important landscape features in Central Europe but their importance for wildlife is mostly overlooked. Their significance as important habitats for birds is best known; even some European endangered species nest in orchards. However, during the last decades orchards have disappeared due the intensification of agriculture (e.g. intensive orchards) and pressures from land development. In Slovenia, for example, more then 5000 ha of its traditionally used orchards were lost since 1990. During three years author studied the breeding birds in 64 traditional orchards in Slovenia. The most common trees in these orchards there have been apple trees. The most common breeding birds in orchards under study were Tree Sparrow (*Passer montanus*) and Great Tit (*Parus major*). In orchards studied breed up to six species, what in most cases depend from the size of orchards. In general, the densities of breeding bird species depends from the size of orchards studied which is also known from other habitat types. Some tips for the management of traditional orchards are presented.

Keywords: breeding birds, Slovenia, traditional orchards, wildlife.

Introduction

Orchards are in some way similar to forest habitat woods (Vogrin, 1994; 1997a), but because orchards are much more manicured than woods, they are perceived by the common public as less important and less rich in species. Traditionally used orchards have been part of our landscape for hundreds of years and represent a mature cultural relationship with nature from which we can learn (Vogrin, 2004).

Standard cider apples for example were usually spaced at approximately 10 metres, cherry trees were grown 15 m, pears over 20 m. Moreover, fruit tress is also long-living, for example, pears can have a productive life of over 300 years.

Traditional orchards is unique habitats mostly in Central Europe, exist mainly in some regions of Austria, Belgium, the Czech Republic, Croatia, France, Germany, Luxembourg, Slovakia, Switzerland and Slovenia and are as such very important for the conservation of biodiversity in this part of Europe.

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In Europe many studies about wildlife, especially about birds, are concentrated on arable lands and various grasslands (Pain and Pienkowski, 1997, and references therein) in agricultural landscapes. On the other side, fruit orchards which support higher populations of birds than any other arable crop or grassland (Lack, 1992) were studied less intensive or were even neglected. Published information about birds that live in cultural landscape and inhabit traditional orchards is scarce elsewhere. Until now only few systematically gathered data are available, mostly from Germany and Switzerland (e.g. Müller *et al.*, 1988; Brandner, 1989; Stevens, 1992; Vogrin, 1994; 1999; 2003; Erlach, 1996). In general, birds in orchards are poorly known, not studied at all or results based on anecdotal data.

In this paper, based on my own results of bird studies from traditional orchards in Slovenia, where, in some part, orchards are still present and are an important part of the landscape, I will shortly reviews the significance of traditionally used orchards for breeding birds, the structure of its breeding bird communities as well as endangered bird species.

Traditional orchards in Slovenia

Until recently every farm, country house and suburban garden in Slovenia had its own collection of fruit trees. Yet over the last few decades traditional orchards have disappeared largely in the wake of cheap imported fruit, the intensification of agriculture (e.g. intensive orchards) and pressures from land development. In Slovenia more then 5000 ha of traditional orchards were lost since 1990 (Statistični urad Republike Slovenije, 1996; 1997). Trends during last 30 years are also negative (r_S = - 0.52, P < 0.05, n = 15) (Fig. 1).

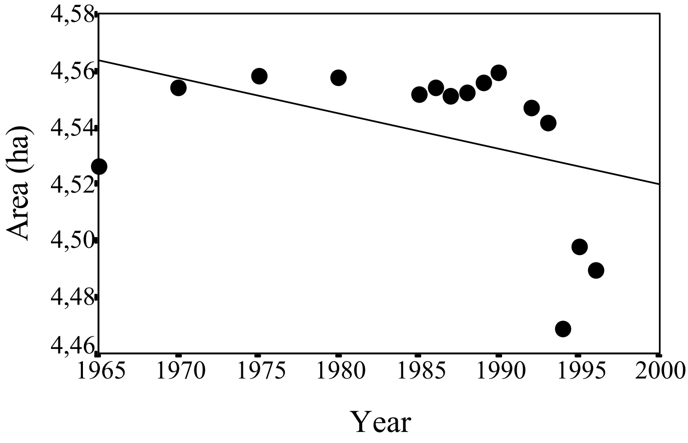

Figure 1. The relationship between orchards area (log) and year in the studied region (r_S = - 0.52, P < 0.05, n = 15).

In contrast in many cities, towns or villages orchards are becoming important for recreation. It can be the focal point for the whole village. Orchards could help to revive an interest in fruit growing, provide a way of sharing knowledge and horticultural skills and stimulate us into growing food for ourselves.

Material and methods

In three years, 1993, 1999 and 2000, I studied breeding birds in 64 traditional orchards in Slovenia with various sizes (see results).

Field investigations were carried out from March to July in during the three years. Breeding birds were counted in the morning after sunrise. In each orchard three checks were usually made with approximately fifteen days intervals throughout the study. Investigations were carried out using the mapping method described by Bibby *et al.* (1992) and Gibbons *et al.* (1996). During the search, all birds encountered were recorded on a simple map of the orchard. Birds were not censused during weather likely to depress bird activity, that is rain and strong winds.

Since data were not normally distributed (Sokal and Rohlf, 1995) I used Spearman correlation coefficient. All statistical tests were performed with the SPSS 13.0 statistical package.

Results and discussion

Birds as example

Most of birds which inhabit traditional orchards in Slovenia are common species. Some endangered species which regularly breed in traditional orchards in Slovenia like Common Kestrel *Falco tinunnculus,* Grey Partridge *Perdix perdix,* Scops Owl *Otus scops,* Little Owl *Athene noctua,* Roller *Coracias garrulus,* Hoopoe *Upupa epops,* Wryneck *Jynx torquilla,* Green Woodpecker *Picus viridis,* Lesser Spotted Woodpecker *Dendrocopus minor,* Common Redstart *Phoenicurus phoenicurus,* Red-backed Shrike *Lanius collurio* and Lesser Grey Shrike *Lanius minor* are also commonly found in orchards across Central Europe. Moreover, for Little Owl and Common Redstart orchards are essential habitat in Slovenia (Vogrin, 1997b), here breed much more then 30% of the entire national population of both species (pers. obs.). This is especially true for the north-eastern part of the country, where both species almost exclusively breed in old traditionally used orchards.

For Slovenia 219 species of breeding birds are documented (Geister, 1995). At least 49 (22%) of these species breed in traditional orchards (Vogrin, 1997; personal observations), despite orchards covers only about 1.3 % of the Slovene area. The number of breeding species in traditional orchards depended heavily on the number of hole nesting species (18 species). These species are also the most endangered ones. Of 49 bird species which have been recorded in traditional orchards in Slovenia as breeding birds, 22 (44.9%) are listed in the Slovenian Red List (Uradni list RS, 2002). Traditional orchards are therefore vital for the conservation of these birds I have considered here, as well for the conservation of other wildlife. Until now, orchards are less important in conservation politics than for example extensive meadows and cereals, because they not support any globally threatened bird species.

Case studies in Slovenia

The study area was located on Mt. Pohorje in North-Eastern Slovenia, and it comprised 30 traditional orchards studied between 1993 and 1994, with a whole area of 22.4 ha, with the average orchard area being 0.74 ha.

Among trees the most frequent were apple-trees that were growing in all traditional orchards where research was carried out followed by pear-tree. 22 birds species were established to breed in traditional orchards. The most frequent breeder was Tree Sparrow *Passer montanus* (nesting in 50% of all orchards studied) followed by Goldfinch *Carduelis carduelis* and Greenfinch *Carduelis chloris*. On the entire area (22.4 ha) 103 breeding pairs were found, i.e. 46 breeding pairs/10 ha (Vogrin, 1999; 2009).

In 2000 I studied the birds in 23 traditional orchards, again on Mt. Pohorje but in different locations as in previous years. The area of all orchards studied varies between 0.03 and 1.03 ha. Again, the most frequent tree was apple-tree. In seventeen orchards (in 6 orchards no species was found) I found 13 different species, the most common was Great Tit *Parus major*, followed by Red-backed Shrike *Lanius collurio*. Number of breeding species was positively correlated with number of trees ($r_s = 0.47$, $P < 0.05$). I was not able to discover which was the reason that birds did not nest in six orchards.

In other study in Central Slovenia in 1999, on an area of 3.75 ha with eleven traditional orchards (average size 0.34 ha) the most frequent tree species was also apple-trees which were present in all orchards. Sixteen species of birds were found to breed. The most frequent breeder was Great Tit *Parus major*. 34.5 breeding pairs were found in all orchards with a density of 92 pairs/10 ha (Vogrin, 2003).

From our studies it is clear that the most common trees in traditional orchards in Slovenia are apple-trees. According to our personal observations the same is the case in most other countries. Densities of breeding birds varies from one pair to more then six, with bird densities depending on the size of orchards and the numbers of trees. Such a rule between area and bird species is well known also for some other habitats. Also in other studies a significant correlation between the size of study areas and densities of breeding birds were found (see for example Luniak 1981; Helle, 1984).

What is also very interesting is that most birds breeding in traditional orchards have in forests greater territories (e.g. Tomialojć and Wesolowski, 1990) then in orchards which are in general smaller then one hectare (see above). Some birds, (e.g. *Parus major*, *Sitta europaea*) which breed in orchards do not forage in unforested habitats. If food supply in these small orchards is limited, birds might be forced to enlarge their feeding areas outside their home orchards, possible into neighbouring orchards, forests or gardens. Breeding in orchards surrounded by open habitats (in most cases, intensive meadows and fields) may be energetically costly. Costs could result from increased food searching time or lower quality food in isolated orchards. Moreover, the exploitation of food resources outside the home orchard may lead to increased time spent searching for prey which could lead to poorer parental care and may possibly increase the risk of predation.

Thus, from the point of conservation it is essential that orchards are not becoming isolated but that we have: 1. more orchards together or, 2. much bigger orchards of more then 5 ha in size (see also Muller *et al.*, 1988; Stevens, 1992) or 3. a mosaics of orchards in a small area, which form "a wood of orchards" with clearings among them.

We should keep in mind that data presented here are only for breeding birds. During the non-breeding season, i.e. during wintering and migration, traditional orchards are also very important habitats for birds. Many species stop here to rest and feed (personal observations).

Importance for other wildlife and other benefits

Many old orchards have occupied the same piece of land for hundreds of years and have undergone minimal, regular regimes of grazing and cutting hay. They have benefited from having few, if any, artificial inputs such as pesticides, herbicides or fertilisers. Consequently they are ideal places for wild flowers, such as various species of orchids. Old orchards are also valuable habitats for hares and various species of wild bees and other insects. Also some amphibians and reptiles, (e.g. Common Toad *Bufo bufo*, Common Frog *Rana temporaria*, Tree Frog *Hyla arborea*, Slow Worm *Anguis fragilis* and Smooth Snake *Coronella austriaca*) found shelter there during the autumn and winter season (personal observations).

Fruits and nuts play an important part in the diet of Red Deer *Capreolus capreolus*, Hedgehog *Erinaceus concolor*, Red Fox *Vulpes vulpes*, Badger *Meles meles* and Fat Dormouse *Glis glis* as well (personal observations). Old apple trees in particular are good hosts for mistletoes and lichens.

Important source of food

Furthermore, for local economises traditional orchards are ideal places for bees and for keeping hives.

Bee keeping in orchards help to pollinate the fruits and provide local people with orchard honey, another source of good food and income. Fruit trees are a source of food for local people and local wild life, and locally grown fruit provides local jobs and reduces transport costs and pollution. Fresh fruit is essential in a healthy diet, providing vitamins and reducing the risk of illness from colds to cancer (Larsen, 1997).

Conservation of traditional orchards

In many cases all old orchards will need replacing the trees which are dead or diseased. It is important not to be over ardent; many fruit trees bear well lying on their sides and make excellent seats. It is important to be planning a succession of new orchards as well as gapping up established ones for the future (see e.g. Rösler 1996). In order to preserve the stock of fruit trees, about 10% of trees must be less than 10 years old. Trimming of trees is also very important for obtaining a good crop of fruits, especially during the first few years.

Conclusion

They are no doubt that traditionally used orchards are important landscape features and special habitats in Central Europe with great importance for wildlife but this importance was mostly overlooked. Traditional orchards are one of the most important habitat in agricultural landscape for birds. In Slovenia, traditional orchards are common, but during last decades pressure of intensification

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RESEARCHES ON LEAF-BEETLES (COLEOPTERA, CHRYSOMELIDAE) FROM THE SOUTHERN AND SOUTH-WESTERN PART OF THE “NATURE 2000” CUSMA SITE (AREA CUSMA-DEALU NEGRU- BUDACULUI VALLEY, DISTRICT OF BISTRIȚA-NĂSĂUD, ROMANIA)

ALEXANDRU CRIŞAN[1,✉]

SUMMARY. A number of 88 species from 38 genera and 8 subfamilies of leaf-beetles were registered in a research made in the Southern ans South-Western part of “Nature 2000” Cusma Site, district of Bistrita-Nasaud, Romania. The results indicate a great diversity of habitats in the area. Three endemic, and a number of 22 rare species were revealed. Chrysomelinae and Halticinae subfamilies so that *Cryptocephalus, Longitarsus* and *Chrysolina* genera were the best represented ones.

Keywords: leaf-beetles, Cusma “Nature 2000”Site, species registered, number, abundance.

Introduction

The present paper deals with the researches developed in the Cusma Site, and constitute a continuation of the researches communicated by us about the Eastern and North-Eastern part of the Cusma Site (Crisan, 2010). Consulting the specific literature (Bobârnac, 1974; Balog *et al.*, 1997; Crişan, 1993a,b, 1994, 1995, 2004, 2006a,b, 2007; Crişan and Teodor, 1994, 2003, 2005: Crişan and Bonea, 1995; Crişan and Druguş, 2001; Crişan and Balint, 2007a,b; Crişan *et al.*, 1998, 1999, 2000, 2003; Fleck, 1905; Gruev *et al.*, 1993; Ieniştea, 1968, 1974; Ieniştea and Negru, 1975; Ilie, 2001; Ilie and Chimisliu, 2000; Konnert-Ionescu, 1963, Maican, 2005; Maican and Serafim, 2001; Marcu, 1927, 1928, 1936, 1957; Negru, 1968, Negru and Roşca, 1967; Roşca,1973, 1974, 1976; Seidlitz, 1891; Szel *et al.*, 1995), we concluded that no data on leaf-beetles in this area were communicated.

Material and methods

In the period June - September 2010 we collected, monthly, leaf-beetles and we made observations in the most illustrative habitats of the Southern and South-Western part of the Cusma Site, the area Cusma- Dealu Negru, Budacului

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Valley. We sampled: mezzo-philous lawns used as hay-lands, situated in the proximity of Cusma village, as well as the bushes bordering these areas; mezzo-xerophilous lawns and clearings used as pastures, placed Eastern from Cusma village; the vegetation bordering the Budacel Valley and the forests of spruce trees in this area; mezo-philous lawns, moist areas, clearings and skirt of beech forests to Dealu Negru and Piatra Corbului areas; lawns, clearings and forest habitats along Budacului Valley, from the beech zone to the high spruce zone.

The collected insect material was put on 85% alcohol and then kept dry till the identification, made in the laboratory using stereo-microscope and appropriate literature (Kaszab, 1962-1971; Kippenberg and Doberl, 1994; Mohr, 1966; Panin, 1951; Petri, 1912; Rozner, 1996; Warkalowsky, 1993, 2003).

Results and discussion

In the collected leaf-beetle material we identified 88 species from 38 genera and 8 subfamilies (Table 1).

There is a great biodiversity on Chrysomelidae family in the research area, both regarding to genera and species identified (46.3% of genera and 15.4% of species comparing with the Romanian leaf-beetle fauna). The 8 identified subfamilies represent also over 61% of the leaf-beetle subfamilies existing in the Romanian fauna (Maican, 2005).

Table 1.

Leaf-beetles from the aria Cuşma-Dealu Negru- Budacului Valley, the Southern and South-Western part of „Nature 2000"site„Cuşma"

Nr. crt.	Subfamily/ Species	Col. date	Nr	Ab. %	Place of capture / Habitat
0	1	2	3	4	5
	I. Donaciinae, Kirby 1837				
1	*Donacia (Donacia) malinovskyi* Ahrens, 1810	16.06	5	0.83	- Piatra Corbului, moist area in a hayland.
2	*Plateumaris (Plateumaris) sericea* (Linnaeus, 1761)	16.06	5	0.83	- Budăcel V., hygr.h. in spruce forest.
		15.06	34	5.67	- Budacului V., moist area in spruce forest.
		14.06	5	0.83	- Dealu Negru, moist area.
		14.07	17	2.83	- Budacului V, hygr. h. in spruce forest.
		15.07	2	0.33	- Budăcel V., hygr.h. in spruce forest
3	*Plateumaris (Juliusiana) braccata* (Scopoli, 1772)	15.06	2	0.33	- Budacului V., moist lawns in mixture species forest.
	II. Criocerinae, Latreille, 1807				
4	*Oulema (Haspidolema) galleciana* (Heyden, 1870)	16.06	1	0.17	- Budăcel V., hygr.h. in spruce forest.
		16.06	1	0.17	- Budacului V, hygr. h. in spruce forest.
5	*Oulema (Haspidolema) erichsoni* (Suffrian, 1841)	15.06	1	0.17	- Piatra Corbului, mezz. lawn.
6	*Oulema (Oulema) melanopus* (Linnaeus, 1758)	14.07	1	0.17	- Dealu Negru, mezz. h.
		15.07	2	0.33	- Budăcel V., h. in spruce forest.
		24.08	1	0.17	- Dealu Negru, mezz. h.

Nr. crt.	Subfamily/ Species	Col. date	Nr	Ab. %	Place of capture / Habitat
0	1	2	3	4	5
	III. Clytrinae, Kirby, 1837				
7	*Labidostomis longimana* (Linnaeus, 1761)	14.06	9	1.50	- W.of Cuşma, mezz. hayland
		15.06	19	3.17	- Piatra Corbului,mezz.hayland
		14.07	12	2.00	- Piatra Corbului,mezz.hayland.
		14.07	2	0.33	- Dealu Negru, mezz.hayland.
		16.07	1	0.17	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
		16.07	1	0.17	- W.of Cuşma, mezz. hayland.
8	*Clytra laeviscula*, Ratzenberg, 1837	14.06	1	0.17	- W.of Cuşma, mezz.hayland.
		16.06	1	0.17	- Piatra Corbului,mezz.hayland.
9	*Smaragdina aurita* (Linnaeus, 1767)	14.06	1	0.17	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
		14.07	2	0.33	- Piatra Corbului, bushes in a mezz. hayland
10	*Smaragdina flavicollis* (Charpentier, 1825)	16.07	1	0.17	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
11	*Smaragdina salicina* (Scopoli, 1763)	14.07	1	0.17	- Piatra Corbului,mezz.hayland.
	IV. Cryptocephalinae, Gyllenhal, 1813				
12	*Cryptocephalus (Burlinius) vittula* Suffrian 1848	15.06	1	0.17	- Piatra Corbului,mezz.hayland.
		06.08	1	0.17	- Piatra Corbului,mezz.hayland.
13	*Cryptocephalus (Burlinius) populi* Suffrian, 1848	06.08	5	0.83	- Budacului V., mezz. lawn in a choped mixed species forest
14	*Cryptocephalus (Burlinius) bilineatus* (Linnaeus, 1767)	14.06	4	0.67	- W.of Cuşma, mezz. lawn.
		15.06	3	0.50	- Piatra Corbului, mezz.lawn.
		14.07	2	0.33	- Piatra Corbului, mezz. lawn.
		06.08	1	0.17	- Piatra Corbului, mezz. lawn.
15	*Cryptocephalus (Cryptocephalus) hypochoeridis* (Linnaeus, 1758)	14.06	17	2.83	- W.of Cuşma, mezz. lawn.
		14.06	7	1.17	- E.of Cuşma, mezzoxer. clearing on a S.exposed hill.
		14.06	4	0.67	- Dealu Negru, mezz. hayland
		15.06	2	0.33	- Budacului V., mezz. h. in mixed species forest skirts.
		15.06	5	0.83	- Piatra Corbului, mezz. lawn.
		16.06	5	0.83	- Budăcel V., clearings in spruce forest.
		16.06	2	0.83	- Budăcel V., clearings in spruce forest.
		14.07	1	0.17	- Budacului V., clearings in spruce forest
		14.07	7	1.17	- Piatra Corbului, mezz.hayland
		14.07	3	0.50	- Dealu Negru, mezz. hayland.
		15.07	4	0.67	- Budăcel V.,h.in spruce forest.
		16.07	4	0.67	- W.of Cuşma, mezz. lawn.
		06.08	1	0.17	- Piatra Corbului, mezz. lawn.
		24.08	1	0.17	- Dealu Negru, mezz.hayland.
16	*Cryptocephalus (Cryptocephalus) bipunctatus* (Linnaeus, 1758)	14.06	2	0.33	- W.of Cuşma, mezz. lawn.
		14.06	3	0.50	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
		14.06	1	0.17	- Dealu Negru, mezz. hayland.
		15.06	1	0.17	- Piatra Corbului, mezz. lawn.
		14.07	1	0.17	- Piatra Corbului, mezz. lawn.
17	*Cryptocephalus (Cryptocephalus) sericeus* (Linnaeus, 1758)	14.06	5	0.83	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
		14.06	1	0.17	- W.of Cuşma, mezz. lawn.
		15.06	6	1.00	- Piatra Corbului, mezz. lawn.
		14.07	2	0.33	- Dealu Negru, h.in a lawn.
		14.07	1	0.17	- Piatra Corbului,mezz.hayland.
		14.07	1	0.17	- W.of Cuşma, mezz. hayland.
		25.08	2	0.33	- Piatra Corbului,mezz.hayland.

Nr. crt.	Subfamily/ Species	Col. date	Nr	Ab. %	Place of capture / Habitat
0	1	2	3	4	5
18	*Cryptocephalus (Cryptocephalus) violaceus* Laicharting, 1781	15.06	5	0.83	- Piatra Corbului,mezz.hayland.
19	*Cryptocephalus (Cryptocephalus) moraei* (Linnaeus, 1758)	14.06	1	0.17	- Dealu Negru, mezz. lawn.
		15.06	1	0.17	- Piatra Corbului, mezz. lawn.
		14.07	9	1.50	- Dealu Negru,mezz.hayland.
		15.07	1	0.17	- Budăcel V.,h.in spruce forest.
		16.07	1	0.17	- W.of Cuşma, mezz.hayland.
		24.08	1	0.17	- Dealu Negru, mezz.hayland.
20	*Cryptocephalus (Cryptocephalus) vittatus* Fabricius, 1775	15.06	16	2.67	- Piatra Corbului, mezz. lawn.
		14.07	5	0.83	- Piatra Corbului, mezz. lawn.
		24.08	1	0.17	- Dealu Negru, mezz. hayland.
21	*Cryptocephalus (Cryptocephalus) sexpunctatus* (Linnaeus, 1758)	14.07	1	0.17	- Budacului V., h. in spruce forest clearings.
22	*Cryptocephalus (Cryptocephalus) biguttatus* (Scopoli, 1763)	15.06	1	0.17	- Piatra Corbului, mezzohygr. area in a hayland.
	V. Chrysomelinae, Latreille, 1802				
23	*Chrysolina (Sphaeromela) varians* (Schaller, 1783)	14.06	1	0.17	- W.of Cuşma, mezz. lawn.
		14.06	2	0.33	- Dealu Negru, mezz.hayland.
		16.06	2	0.33	- Budăcel V., hygr.h. in a spruce forest.
		16.06	6	1.00	- Piatra Corbului,mezz.hayland.
		16.06	3	0.50	- Budacului V.,h.in spruce forest.
		14.07	2	0.33	- Piatra Corbului,mezz.hayland.
		14.07	2	0.33	- Budacului V.,h in spruce forest.
		15.07	3	0.50	- Budăcel V.,h.in spruce forest.
		16.07	3	0.50	- W.of Cuşma, mezz. lawn.
		06.08	2	0.33	- Piatra Corbului,mezz.hayland.
		24.08	1	0.17	- Dealu Negru, mezz.hayland.
		25.08	1	0.17	- Piatra Corbului,mezz.hayland.
		25.08	1	0.17	- Cuşmei peak, subalpine lawn with *Festuca*.
24	*Chrysolina (Ovostoma) globipennis* (Suffrian, 1851)	15.06	1	0.17	- Budacului V., moist lawns in mixture species forest.
25	*Chrysolina (Menthastriella) herbacea* (Duftschmid, 1825)	14.06	2	0.33	- Dealu Negru, h.,spruce forest.
		14.06	6	1.00	- Dealu Negru, h.in moist area..
		15.06	2	0.33	- Budacului V., moist lawns in mixture species forest.
		25.08	1	0.17	- Cuşmei peak, h.in moist area.
26	*Chrysolina (Chalcoidea) marginata* (Linnaeus, 1758)	14.06	2	0.33	- W.of Cuşma, mezz. lawn.
27	*Chrysolina (Sphaerochrysolina) rufa* (Duftschmid, 1825)	16.06	1	0.17	- Piatra Corbului,mezzohygr. area in a hayland.
		05.08	1	0.17	- Dealu Negru, h. in moist area.
28	*Chrysolina (Erythrochrysa) polita* (Linnaeus, 1758)	14.06	1	0.17	- Dealu Negru, h. in moist area.
		06.08	1	0.17	- Budacului V., clearings in choped mixed forest.
29	*Chrysolina (Ovostoma) olivieri* (Bedel, 1892)	14.07	3	0.50	- Piatra Corbului,mezz.hayland.
		24.08	1	0.17	- Dealu Negru, mezz. hayland
30	*Chrysolina (Colaphosoma) sturmi* (Westhoff, 1882)	24.08	1	0.17	- Dealu Negru, clearings in a beech forest.
31	*Chrysolina (Fastuolina) fastuosa* (Scopoli, 1763)	16.06	4	0.67	- Piatra Corbului, mezzhygr. area in a hayland.
		16.06	2	0.33	- Budacului V., hygr.h. in spruce forest.
		14.07	2	0.33	- Dealu Negru,h. in moist area.
		15.07	1	0.17	- Budăcel V.,h.in spruce forest.
		06.08	1	0.17	- Budacului V.,choped forest.
		24.08	1	0.17	- Dealu Negru, h. in moist area.

Nr. crt.	**Subfamily/ Species**	**Col. date**	**Nr**	**Ab. %**	**Place of capture / Habitat**
0	1	2	3	4	5
32	*Oreina (Allorina) coerulea* (Olivier, 1790)	14.06	18	3.33	- W.of Cuşma, mezz. lawn.
		16.06	13	2.17	- Piatra Corbului, mezzohygr. area in a hayland.
		14.07	2	0.33	- Piatra Corbului, mezzohygr. area in a hayland.
		16.07	4	0.67	- W.of Cuşma, mezz. lawn.
33	*Oreina (Intricatorina) intricata* (Germar, 1824) *anderschi* (Duftschmid, 1825)	14.06	12	2.00	- Dealu Negru, moist area in a spruce forest.
		15.06	1	0.17	- Budacului V., moist lawns.
		16.06	6	1.00	- Piatra Corbului, h.in a mezzohygr. area of a hayland.
		14.07	1	0.17	- Piatra Corbului,mezz.hayland.
		06.08	1	0.17	- Budacului V.,choped forest.
		25.08	3	0.50	- Piatra Corbului, h.in a mezzohygr. area of a hayland
34	*Gastrophysa viridula* (De Geer, 1775)	15.07	1	0.17	- Budăcel V., h.in spruce forest.
35	*Gastrophysa polygoni* (Linnaeus, 1758)	15.07	1	0.17	- Budăcel V., hygr. h. in spruce forest.
36	*Phaedon (Sternoplatys) segnis* Weise 1884	16.06	1	0.17	- Budăcel V., hygr. h. in a spruce forest.
37	*Sclerophaedon carpathicus* Weise 1875	16.06	7	1.17	- Budăcel V., hygr. h. in a spruce forest.
		14.07	1	0.17	- Budacului V., hygr. h. in a spruce forest.
38	*Sclerophaedon carniolicus* (Germar, 1824)	14.06	1	0.17	- Dealu Negru, moist area.
		14.07	2	0.33	- Budacului V., hygr. h. in a spruce forest.
39	*Hydrothassa marginella* (Linnaeus, 1785)	14.07	1	0.17	- Budacului V., hygr. h. in a spruce forest.
		24.08	1	0.17	- Dealu Negru, h.in a moist area
40	*Linaeidea (Linaeidea) aenea* (Linnaeus, 1758)	14.06	2	0.33	- Cuşma, on *Salix* and *Alnus*.
		16.07	1	0.17	- Cuşma, on *Salix* and *Alnus*.
41	*Chrysomela (Stricherus) cuprea* (Fabricius, 1775)	25.08	1	0.17	- Piatra Corbului, h. in a beech forest.
42	*Chrysomela (Chrysomela) populi* Linnaeus, 1758	14.06	4	0.67	- W.of Cuşma, on *Salix caprea*.
		16.07	1	0.17	- W.of Cuşma, on *Salix caprea*.
		25.08	2	0.33	- Cuşmei peak, on *Salix caprea*.
43	*Gonioctena (Goniomena) interposita* (Frtanz et Palmen, 1950)	14.07	2	0.33	- Budacului V., h. in a spruce forest.
44	*Timarcha (Timarcha) regulosa* Herrich-Schaeffer, 1838	16.06	2	0.33	- Piatra Corbului, mezzohygr. area in a hayland.
	VI. Galerucinae, Latreille, 1802				
45	*Galerucella (Neogalerucella) calmariensis* (Linnaeus, 1767)	15.06	1	0.17	- Budacului V., moist lawns in mixture species forest.
46	*Galerucella (Neogalerucella) pusilla* (Duftschmid, 1825)	25.08	3	0.50	- Piatra Corbului,mezz.hayland.
47	*Lochmea capreae* (Linnaeus, 1758)	16.06	1	0.17	- Budăcel V., on *Salix caprea*.
		15.07	1	0.17	- Budăcel V., on *Salix caprea*.
		25.08	1	0.17	- Cuşmei peak, on *Salix caprea*.
48	*Galeruca (Galeruca) tanaceti* (Linnaeus, 1758)	14.06	11	1.83	- W.of Cuşma, mezz. lawn.
		14.06	1	0.17	- E.of Cuşma,forest skirts lawn.
		14.06	2	0.33	- Dealu Negru, hygr. lawn.
		16.06	5	0.83	- Piatra Corbului,mezz.hayland.
		14.07	1	0.17	- Budacului V.,h.,spruce forest.
		16.07	5	0.83	- W.of Cuşma, mezz. lawn.
		25.08	1	0.17	- Cuişmei peak, hygr. area.
49	*Galeruca (Galeruca) pomonae* (Scopoli, 1763)	15.07	1	0.17	- Dealu Negru, mezz. hayland.
		24.08	1	0.17	- Dealu Negru, mezz. hayland.
50	*Calomicrus circumfusus* (Marsham, 1802)	14.06	2	0.33	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.

Nr. crt.	**Subfamily/ Species**	**Col. date**	**Nr**	**Ab. %**	**Place of capture / Habitat**
0	1	2	3	4	5
51	*Luperus viridipennis* Germar, 1824	15.06	1	0.17	- Piatra Corbului,on the bushes in a mezz. hayland.
52	*Luperus luperus* Sulzer, 1776	14.06	1	0.17	- Cuşma, clearings in a spruce forest.
	VII. Halticinae Newman 1854				
53	*Aphthona stussineri* Weise, 1888	14.06	2	0.33	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
54	*Aphthona semicyanea* Allard, 1859	13.07	1	0.17	- Dealu Negru, mezz. h..
55	*Aphthona venustula* (Kutschera, 1861)	24.08	1	0.17	- Dealu Negru, mezz.hayland.
56	*Longitarsus (Longitarsus) apicalis* (Beck, 1817)	14.07	1	0.17	- Piatra Corbului,mezz.hayland.
		25.08	1	0.17	- Piatra Corbului,mezz.hayland.
57	*Longitarsus (Longitarsus) membranaceus* (Foudras, 1860)	14.07	1	0.17	- Piatra Corbului,mezz.hayland.
58	*Longitarsus (Longitarsus)succineus* (Foudras, 1860)	14.06	1	0.17	- Dealu Negru, moist area.
59	*Longitarsus (Longitarsus) rubellus* (Foudras, 1860)	14.06	2	0.33	- Dealu Negru, moist area.
		16.06	1	0.17	- Budacului V., h., in a spruce forest.
60	*Longitarsus (Longitarsus) symphyti* Heikertinger, 1912	24.08	1	0.17	- Dealu Negru, on *Symphitum cordatum* in a spruce forest.
61	*Longitarsus (Longitarsus) brunnaeus* (Duftschmid, 1825)	14.06	2	0.33	- W. of Cusma, mezz. hayland.
		05.08	6	1.00	- Dealu Negru, mezz. hayland.
62	*Longitarsus (Longitarsus) pratensis* (Panzer, 1794)	05.08	4	0.67	- Dealu Negru, mezz. hayland.
		06.08	1	0.17	- Piatra Corbului, mezz. lawn.
63	*Longitarsus (Longitarsus) lycopi* (Foudras, 1860)	05.08	1	0.17	- Dealu Negru, mezz. hayland.
		24.08	1	0.17	- Dealu Negru, mezz. hayland.
64	*Longitarsus (Testergus) anchusae* (Paykull, 1799)	14.07	1	0.17	- Piatra Corbului, mezz. hayland.
65	*Altica quercetorum* Foudras, 1860, saliceti (Weise, 1888)	15.07	1	0.17	- Budăcel V., h. in a spruce forest
66	*Altica carduorum* (Guerin-Meneville, 1858)	15.06	1	0.17	- Budacului V., moist area.
		16.07	1	0.17	- W.of Cuşma, mezz. lawn.
67	*Batophila fallax* Weise, 1888	14.06	2	0.33	- Dealu Negru, hygr. h. in a spruce forest
		16.06	1	0.17	- Budăcel V.,h.in spruce forest.
		16.06	16	2.67	- Budacului V., hygr. h. in spruce forests.
		15.07	1	0.17	- Budăcel V.,h.in spruce forest.
68	*Asiorestia femorata* (Gyllenhal, 1813)	16.06	2	0.33	- Budăcel V.,h.in spruce forest.
		16.06	2	0.33	- Budacului V.,h. in spruce forest.
69	*Asiorestia transsylvanica* (Fuss, 1864)	15.06	3	0.50	- Budacului V., mezz. lawn.
		16.06	1	0.17	- Budăcel V.,h.in spruce forest.
		15.07	1	0.17	- Budăcel V.,h.in spruce forest.
		06.08	2	0.33	- Budăcel V., spruce forest clearings.
70	*Asiorestia cyanipennis* (Kutschera, 1860)	16.06	1	0.17	- Budăcel V.,h.in spruce forest.
71	*Asiorestia ferruginea* (Scopoli, 1763)	14.06	1	0.17	- E.of Cuşma, mezzoxer. pasture on a S. exposed hill.
		15.06	5	0.83	- Piatra Corbului, mezz. lawn.
		14.07	2	0.33	- Piatra Corbului, mezz. lawn.
		24.08	1	0.17	- Dealu Negru, mezz.hayland.
		25.08	1	0.17	- Piatra Corbului, mezz. lawn.
72	*Derocrepis rufipes* (Linnaeus, 1761)	14.06	5	0.83	- W.of Cuşma, mezz. lawn.
		15.07	2	0.33	- Budăcel V., spruce clearings.
		16.07	2	0.33	- W.of Cuşma, mezz. lawn.
73	*Crepidodera aurata* (Marsham, 1802)	14.06	1	0.17	- W.of Cuşma, on *Salix caprea*.
		14.06	1	0.17	- Dealu Negru, on *Salix caprea*.
		16.06	1	0.17	- Budăcel V., on *Salix caprea*.
		15.07	2	0.33	- Budăcel V., on *Salix caprea*.
		25.08	1	0.17	- Piatra Corbului, on *Salix* sp.
		25.08	1	0.17	- Cuşmei peak,on *Salix caprea*.

Nr. crt.	Subfamily/ Species	Col. date	Nr	Ab. %	Place of capture / Habitat
0	1	2	3	4	5
74	*Crepidodera aurea* (Geoffroy,1875)	15.06	3	0.50	- W.of Cuşma, pe *Salix caprea*.
		16.07	2	0.33	- W.of Cuşma, pe *Salix caprea*.
75	*Minota carpathica* Heikertinger, 1911	15.07	1	0.17	- Budăcel V., h. in a spruce forest.
76	*Mantura chrysanthemi* (Koch, 1803)	14.07	1	0.17	- Budacului V., h. in a spruce forest
77	*Chaetocnema (Tlanoma) semicoerulea* (Koch, 1803)	15.06	1	0.17	- Dealu Negru, h. in a mezz. hayland.
78	*Chaetocnema (Chaetocnema) aerosa* (Letzner, 1846)	24.08	1	0.17	- Dealu Negru, h. in a mezz. hayland.
79	*Sphaeroderma testaceum* (Fabricius, 1775)	14.06	1	0.33	- W.of Cuşma, mezz. hayland.
80	*Sphaeroderma rubidum* (Graells, 1858)	24.08	1	0.17	- Dealu Negru, mezz. lawn.
		25.08	1	0.17	- Cuşmei peak, mezz. skirts h.
81	*Apteropeda splendida* Allard, 1860	16.06	1	0.17	- Budăcel V., hygr. h. in a spruce forest.
82	*Mniophila muscorum* (Koch, 1803)	16.06	1	0.17	- Budacului V., by sifting beech dead leaves.
83	*Dibolia (Dibolia) cryptocephala* (Koch, 1803)	15.06	1	0.17	- Piatra Corbului, mezz. hayland.
84	*Dibolia (Dibolia) foersteri* Bach, 1859	14.06	1	0.17	- W.of Cuşma, mezz. hayland.
		15.06	2	0.33	- Piatra Corbului,mezz.hayland.
85	*Psylliodes (Psylliodes) isatidis* Heikertinger, 1912	14.07	1	0.17	- Budacului V., moist area in a spruce forest
	VIII. Cassidinae, Gyllenhal, 1813				
86	*Cassida (Odontionycha) viridis* Linnaeus, 1758	15.06	4	0.67	- Budacului V., hygr. h..
		15.06	3	0.50	- Piatra Corbului, moist area.
		16.06	6	1.00	- Budăcel V.,h.in spruce forest
		13.07	1	0.17	- Dealu Negru, hygr. h..
		14.07	1	0.17	- Piatra Corbului, moist area
87	*Cassida (Cassida) vibex* Linnaeus, 1767	25.08	1	0.17	- Piatra Corbului, mezz. hayland.
88	Cassida (Cassida) lineola Creutzer, 1799	25.08	1	0.17	- Piatra Corbului, mezz. hayland

***Abreviations**: **A**. = relative abundance; **Col. date**=collecting date; **Nr** = number of individuals; E.= East; h.= herbs; hygr.= hygrophilous; mezz.= mezzophilous; mezzohygr.= mezzo-hygrophilous; V.= Valley; W.= West.*

The great number of species of Chrysomelinae and Halticinae subfamilies (Fig.1) indicate the presence of a great number of hygrophilous and mezzo-hygrophilous habitats in the research area, but also the presence of xerophilous ones, as a consequence of the diversity in the relief of the area, as well as of the richness of the hydrographical net.

Concerning to the genera (Fig.2), the great number of species of *Cryptocephalus* and *Longitarsus*, on the one hand, as well as of *Chrysolina*, on the other hand, support also the considerations above made on the characteristics of the area.

The fact that 29 of the 38 registered genera have had only one or two species indicate the real value of the researched area concerning the diversity of habitats and specific micro-local conditions. More of these, some genera as *Hydrotassa, Calomicrus, Apteropeda* and *Mniophila* were registered by us for the first time, although we developed researches on the leaf-beetle group in diverse parts of the country (see the references!), this indicating that Cusma site has habitats with particular ecological characteristics.

A lot of the registered species are rare from the Romanian fauna: *Donacia (Donacia) malinowskyi, Oulema (Haspidolema) erichsoni, Smaragdina flavicollis, Cryptocephalus (Cryptocephalus) sexpunctatus, Chrysolina (Ovostoma) globipennis, Chrysolina (Calcoidea) marginata, Chrysolina (Colaphosoma) sturmi, Phaedon (Sternoplatis) segnis, Sclerophaedon carniolicus, Hydrothassa marginella, Chrysomela (Strikerus) cuprea, Galerucella (Neogalerucella) pusilla, Calomicrus circumfusus, Aphthona semicyanea, Asiorestia cyanipennis, Derocrepis rufipes, Mantura chrysanthemi, Sphaeroderma testaceum, Apteropeda splendida, Mniophila muscorum, Dibolia (Dibolia) cryptocephala, Psylliodes (Psylliodes) isatidis*. These, as well as some endemical ones: *Sclerophaedon carpathicus, Asiorestia transsylvasnica, Minota carpathica,* constitute great reasons and arguments for the status of „Nature 2000" site for the Cusma zone, and justify the intensification of the activities of protection and conservation of the biodiversity in this site.

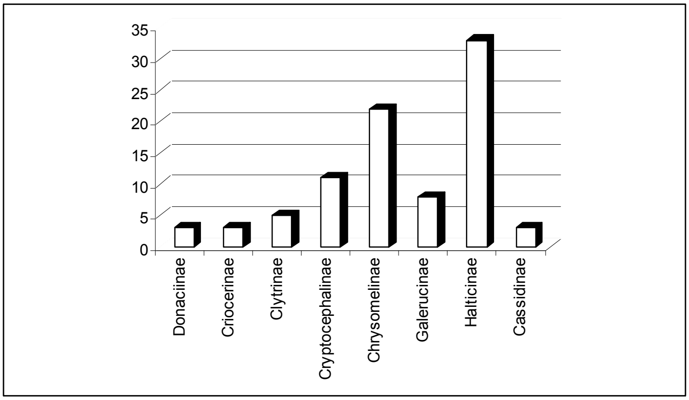

Fig.1. Number of species of leaf-beetles in different subfamilies identified in the Southern and South-Western part of Cusma "Nature 2000"site.

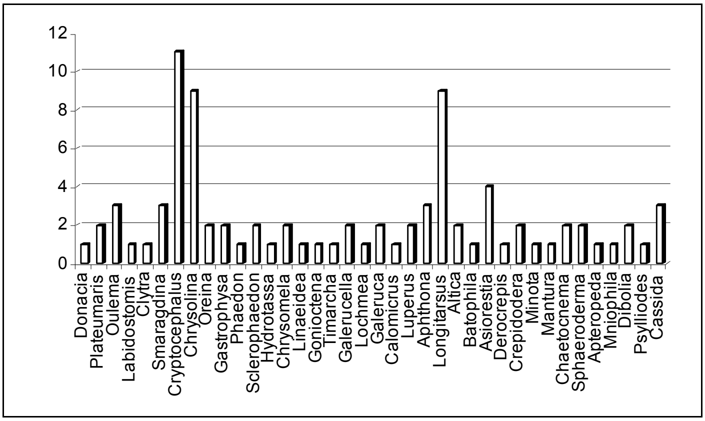

Fig. 2. Number of species in the leaf-beetle genera identified in the Southern and South-Western part of the "Nature 2000"Cusma site.

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AQUATIC INVERTEBRATE DRIFT IN SOMEŞUL CALD RIVER WITH SPECIAL REGARDS ON EPHEMEROPTERA TAXA

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SUMMARY. Drift of aquatic invertebrates were studied in the River Someşul Cald. Samples were collected every 3 hours on the 10th and 11th August 2005 during a 24 hour period. Specimens were sorted into higher taxonomic units and counted. Mayflies (Ephemeroptera) were identified to the lowest level possible and measured. According to the results both the number and size of mayflies entering the drift increased during the night. Principal Component Analysis (PCA) and Canonical Correspondence Analysis (CCA) ordination sustains that light is an important factor influencing the drift behaviour of mayflies. *Rhithrogena circumtatrica* was cited for the first time in Romania in the present paper.

Keywords: aquatic communities, drift, Ephemeroptera (mayflies), the Someşul Cald River.

Introduction

The simplest definition of the drift was given by downstream transport of aquatic organisms, due to water current. The literature presents three main types of drift: constant, catastrophic and behavioral (Waters, 1972).

Even if the first reports on drift date back to the beginning of the 20th century, this phenomenon caught the scientists' attention only after 1950, when Müller (1954) published his results on Swedish rivers. Since then, numerous papers focused on the main environmental factors influencing the drift (Waters, 1972; Statzner *et al.*, 1984; Brittain and Eikeland, 1988; Allan, 1995; Boyero *et al.*, 2005; James *et al.*, 2008; Neale *et al.*, 2008, Tonkin *et al.*, 2009; Pond, 2010)

Ephemeroptera larvae are omnipresent components of river aquatic communities (Allan, 1995) and for many mayfly species active movement in the water column or drift represents the main form of dispersal (Elliott, 2002). The rate of drift in mayflies is directly correlated with food foraging (Kohler, 1985); it can influence the movement of individuals (Peckarsky and McIntosh, 1998), the population abundance (Forrester, 1994) and the community structure (Diehl *et al.*, 2000). Moreover, the movement of mayflies can influence the abundance of predators (Englund, 1997; Forrester *et al.*, 1999; Diehl *et al.*, 2000). Many studies emphasize that mayfly drift

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density has low values during the day but increases severely after sunset (Elliott, 1967; Waters, 1972; Müller, 1974). Some authors consider that individuals tend to be disorientated in the absence of light (Hughes, 1966; Statzner and Mogel, 1985). Others suggest that the individuals entering the drift might be genetic inferior to those living in benthic communities or that they are weakened by infections (Minshall and Petersen, 1985). However, these theories do not explain the diurnal periodicity of the drift in Ephemeroptera communities. This periodicity might be caused by the avoidance of predators, especially fish that are visual predators (Allan, 1995; Huhta *et al.*, 2000). Thus, by decreasing their movements in the water column during daytime, mayfly larvae lower their risk to become food for predators. Some authors have proved that the presence of chemical cues from trout might affect the diurnal periodicity of the drift of mayflies; they also showed that this effect was different depending on the size of the larvae (McIntosh and Peckarsky, 1996; Tikkanen *et al.*, 1996; McIntosh *et al.*, 1999). McIntosh *et al.* (2002) concluded that the risk of capture of the Ephemeroptera larvae by fish was 4 to 5 times greater during the day. They have also proved that the predator density has a significant effect on the drift of some species and that larger larvae were more vulnerable to predators both during the day and during the night.

The purpose of the present study is to prove that not only the number but also the size of mayflies entering the drift increased during the night.

Material and Methods

Sampling site is located in the upper part of the Someşul Cald River in the mountain area with low human impact. Drift samples were collected every 3 hours during 24 hours, on the 10th and 11th of August 2005, using a 250 µm mesh net with a metallic frame (1m long and 1m high). Two such nets were used, one located 1.5m from the left bank, the other only 1m from the right bank and the distance between nets was 2m. The samples were collected at 6:00, 9:00, 12:00, 15:00, 18:00, 21:00, 24:00 and 3:00 o'clock. Each sampling lasted for 30 minutes. In order to have a more accurate species inventory quantitative macroinvertebrate samples have been collected using a Surber benthic sampler with a 250 µm mesh net.

All samples were preserved in 38% formaldehyde. Water temperature and the quantity of dissolved oxygen were measured using an YSI Model 52 oxygen-meter; the air temperature was also recorded.

The biological material was sorted in the lab under a SMZ645 Nikon stereo microscope. Organisms belonging to different taxonomic groups were transferred in 70% alcohol. Ephemeroptera taxa were identified to the lowest level possible using the following keys: Müller-Liebenau, 1969; Studemann *et al.*, 1992; Soldan and Landa, 1999.

Mayfly species were measured and individuals were divided into several size classes, ranging from 0.5 to 12 mm (Table 3). Their length from the labrum to the basis of the cerci was considered.

Several ecological indices were calculated: the percentage abundance and the density of the taxonomical groups, together with the frequency of appearance of mayfly taxa. In order to visualize the relationships between the physical and chemical parameters and the Ephemeroptera communities, multivariate analyses were carried out: Principal Component Analysis (PCA) and Canonical Correspondence Analysis (CCA), using the XLSTAT program (trial version, www.xlstat.com).

Tables 1, 2 and 3 present the list of abbreviations used for this paper.

Table 1.

List of abbreviations used for samples

Code	Sample	Code	Sample
D9	Drift sample collected from 9:00 to 9:30	D24	Drift sample collected from 24:00 to 24:30
D12	Drift sample collected from 12:00 to12:30	D3	Drift sample collected from 3:00 to 3:30
D15	Drift sample collected from 15:00 to 15:30	D6	Drift sample collected from 6:00 to 6:30
D18	Drift sample collected from 18:00 to 18:30	B	Benthos samples
D21	Drift sample collected from 21:00 to 21:30		

Table 2.

List of abbreviations used for Ephemeroptera taxa

Code	Taxa	Code	Taxa
Bme	*Baetis melanonyx* (Pictet 1843)	Eas	*Epeorus assimilis* Eaton 1885
Bmu	*Baetis muticus* (Linnaeus 1758)	Rci	*Rhithrogena circumtatrica* Sowa & Soldan 1986
Brh	*Baetis rhodani* (Pictet 1843)	Rsp1	*Rhithrogena* sp1
Bve	*Baetis vernus* Curtis 1834	Rcfca	*Rhithrogena carpatoalpina* Klonowska, Olechowska, Sartori & Weichselbaumer 1987
Bsp.	*Baetis* sp.	Rsp	*Rhithrogena* sp.
Sig	*Serratella ignita* (Poda 1761)	Hc	*Habroleptoides confusa* Sartori & Jacob 1986
Ecsp	*Ecdyonurus* sp.	Subima	subimagoes

Table 3.

List of abbreviations used for size classes of Ephemeroptera

Size class	Body length, cerci and antenna excluded (L)	Size class	Body length, cerci and antenna excluded (L)
I	(0.5 – 1.0 mm)	VII	(6.1 – 7.0 mm)
II	(1.1 – 2.0 mm)	VIII	(7.1 – 8.0 mm)
III	(2.1 – 3.0 mm)	IX	(8.1 – 9.0 mm)
IV	(3.1 – 4.0 mm)	X	(9.1 – 10.0 mm)
V	(4.1 – 5.0 mm)	XI	(10.1 – 11.0 mm)
VI	(5.1 – 6.0 mm)	XII	(11.1 -12.0 mm)

Results and discussion

Water temperature ranged from a minimum of 7.6°C at 3 a.m. to 10.2°C at 3 p.m. The variation of the temperature between night and day was 2.6°C. The air temperature recorded a broader variation between night and day, ranging from the lowest value of 6°C during night to a maximum of de 17.5°C at 3 p.m. Thus, the maximum water and air temperatures were recorded at 3 p.m., while the minimum values were observed at 3 a.m. in the water and at 6 a.m. in the atmosphere (Fig. 1). The quantity of dissolved oxygen ranged between 6.66 mg/l, recorded at 6 a.m. to a maximum of 8.57 mg/l at 3 p.m. (Fig. 1). Thus, oxygen consumption by algae and animals during the night led to decreases in its quantity in the water. During the day, dissolved oxygen values increased due to photosynthesis.

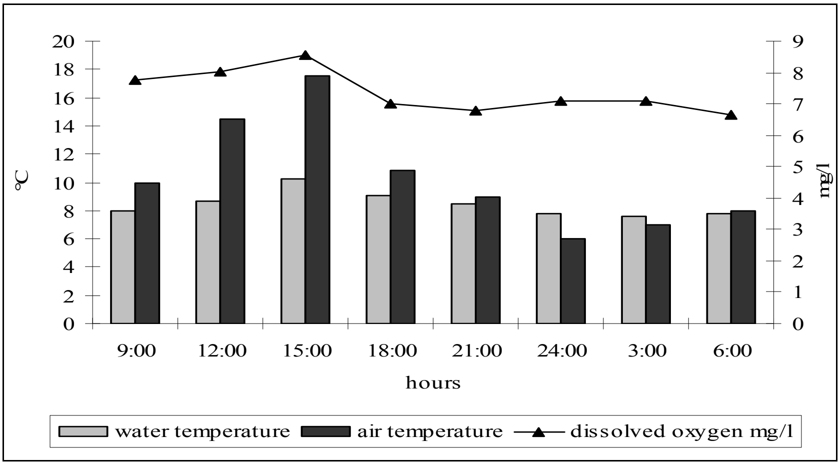

Fig. 1. Water and air temperature (°C) and dissolved oxygen (mg/l) measured at the eight sampling periods from 10 to 11 August 2005

A total of 27399 organisms (Table 4) were present in the drift samples, 16842 (61.5%) of them were Ephemeroptera. The highest number of individuals was recorded at 9 p.m., right after the sunset.

In the benthos samples (Table 4) the mayflies recorded the highest density, reaching 1363 individuals/m^2, which represents 49.5% of the total density of benthic organisms, followed by chironomids (667 individuals/m^2). The lowest density was presented by oligochaets: 4 individuals/m^2. Turbellaria, Nematoda, Amphipoda, Copepoda, Ostracoda and Coleoptera were absent from the benthos samples, but they were present in the drift in low densities.

Table 4.
The total number of organisms present in the drift samples (D9 – D6) and their density in the benthos sample (B)

Sample	**Drift samples** (no of individuals)									**Benthos Samples** (individuals/m^2)
Taxa	**D9**	**D12**	**D15**	**D18**	**D21**	**D24**	**D3**	**D6**	**Total**	**B**
Turbellaria	0	1	0	1	0	0	0	0	2	0
Nematoda	9	25	22	37	28	9	13	17	160	0
Oligochaeta	0	11	7	4	9	9	10	20	70	3.70
Hydrachnidia	21	204	168	142	54	9	9	41	648	88.89
Amphipoda	1	1	3	2	3	1	2	2	15	0
Copepoda	1	6	2	0	5	1	5	1	21	0
Ostracoda	0	0	1	0	0	0	0	0	1	0
Coleoptera	2	0	4	2	2	5	6	2	23	0
Chironomidae	232	1354	1673	855	932	359	433	746	6584	666.67
Diptera - others	34	56	112	86	390	146	132	77	1033	92.59
Ephemeroptera	598	1161	1114	1186	3459	2831	3589	2904	16842	1362.96
Plecoptera	38	141	232	475	393	180	160	214	1833	492.59
Trichoptera	5	14	26	24	9	10	13	66	167	22.22
Total	941	2974	3364	2814	5284	3560	4372	4090	27399	2751.85

Mayflies, together with chironomids represented the dominant groups as percentage abundance (Fig. 2), not only in the drift samples but also in the benthos ones. Mayflies dominated in all samples, except those collected at noon and at 3 p.m., when chironomids exceeded their number, reaching a maximum of 48%. Chironomids were well represented during the night as well, but their abundances did not exceed 19%. In the night samples, the mayflies recorded abundances greater than 70%, with a maximum of 83% at 3 a.m. The abundances of stoneflies (Plecoptera) in the drift samples, recorded a maximum percentage of 15.5% at 6 p.m., while in the benthos samples they recorded 18%. The category "other groups" included the following: Oligochaeta, Turbellaria, Nematoda, Hydrachnidia, Amphipoda, Copepoda, Ostracoda, Coleoptera, other dipterans and Trichoptera. Their percentage abundance (as a whole) ranged between 11% at 9 a.m. and 4% at 3 a.m. In the benthos samples, the category "other taxa" included only Oligochaeta, Hydrachnidia, other dipterans and Trichoptera, and their percentage abundance did not exceed 8%.

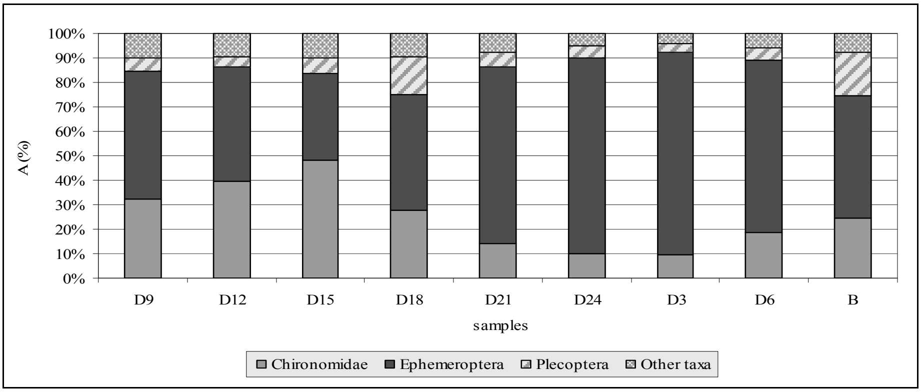

Fig. 2. The percentage abundance (A%) of the main benthic invertebrate groups in the drift (D9-D6) and benthos (B) samples

The number of mayflies captured in the drift samples (Fig. 3), increased gradually starting from 9 a.m., when almost 600 individuals were caught, reaching 3459 at 9 p.m., after sunset. Mayflies recorded high numbers during night too, with a slight decrease at midnight (2831 individuals), with a maximum at 3 a.m. (3589 individuals), and with a subsequent decrease at 6 a.m. (2904 individuals). This model is similar to those described in the literature, where the mayfly drift is known to record two maxima: the first, the most important, occurring in the second half of the night, and the second one occurring after sunset (Müller, 1965; Flecker, 1992). Most of mayfly larvae are active during night. Some of them are pulled away from the substratum and taken by the water current. Some authors (Waters, 1972; McIntosh and Townsend, 1995) consider that this larval drift is more intense during night. Our data showed that from the total number of 16842 mayflies caught in the drift samples, 12783 (75%) were captured at night, and only 4059 during the day.

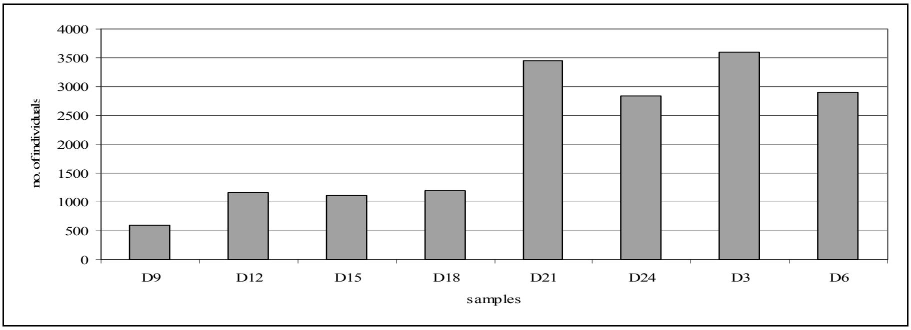

Fig. 3. The number of mayfly individuals in the drift samples

13 Ephemeroptera taxa were identified in the drift samples (Fig. 4). *Baetis melanonyx*, *Baetis* sp. and *Rhithrogena* sp. young instars reached a frequency of 100%. *Baetis rhodani, B. vernus, Serratella ignita, Ecdyonurus* sp.*, Rhithrogena circumtatrica, R.* sp1*, Habroleptoides confusa* and subimagoes exceeded 60%. *Baetis muticus, Epeorus assimilis* and *Rhithrogena cf carpatoalpina* recorded frequency values that did not exceed 40%.

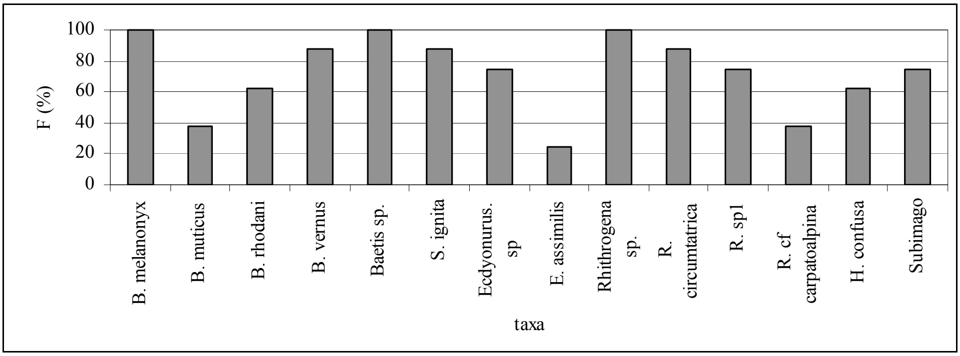

Fig. 4. The frequency (F%) of mayfly species in the drift samples

Baetis muticus, *B. rhodani*, *Epeorus assimilis*, *Rhithrogena cf carpatoalpina* and *Habroleptoides confusa* were present only in the drift samples, with no representatives in the benthos samples (Table 5). They recorded very low percentage abundances compared to other mayfly species, not exceeding 0.3%. Subimagoes were also present only in the drift samples (with percentages not exceeding 0.4%) as transformation from larvae to subimago takes place on the water surface (Studemann *et al.*, 1992). On the other hand, *Baetis melanonyx* exceeded 20%, not only in the benthos samples but also in the drift ones.

Table 5.

The percentage abundance (%) of mayfly species in the drift and benthos samples (D - drift samples; B - benthos samples)

Taxa	*B. melanonyx*	*B. muticus*	*B. rhodani*	*B. vernus*	*Baetis* sp.	*S. ignita*	*Ecdyonurus*. sp.	*E. assimilis*	*R. circumtatrica*	*R.* sp1	*R .cf carpatoalpina*	*Rhithrogena* sp.	*H. confusa*	Subimago
D	24.46	0.02	0.31	0.33	71.46	0.46	0.17	0.02	0.30	1.08	0.02	0.96	0.04	0.37
B	20.62	0.00	0.00	0.29	63.39	1.61	0.81	0.00	4.62	1.91	0.00	6.75	0.00	0.00

In the benthos samples, *Baetis melanonyx* and *Rhithrogena circumtatrica* reached the highest densities (Fig. 6), followed by *Rhithrogena* sp1, *Serratella ignita* and *Ecdyonurus* sp. The lowest density was recorded by *Baetis vernus*, with a total of 4 individuals/m^2. Young larvae of *Baetis* and *Rhithrogena* were also well represented.

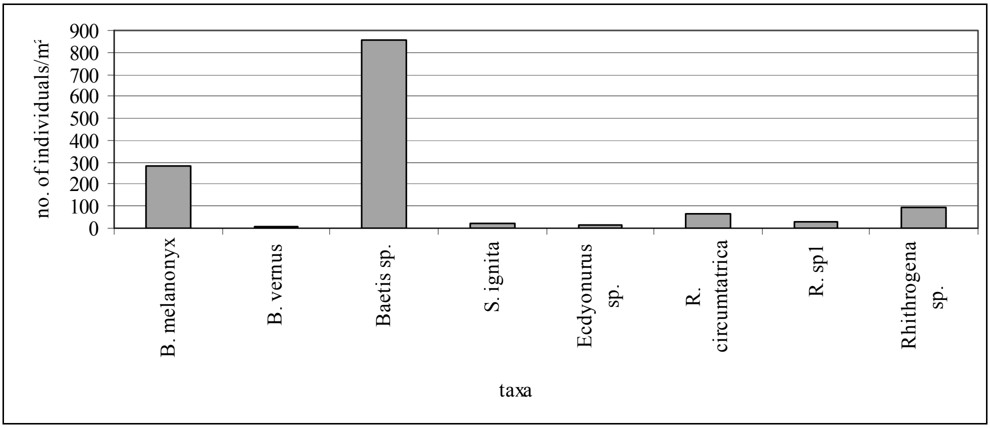

Fig. 6. The density of different mayfly species from the benthos samples (no of individuals/ m^2)

The maximum length was recorded by a subimago. *Baetis* individuals smaller than 2 mm recorded the highest numbers not only in the drift samples, but also in the benthos ones. The high number of young individuals no larger than 2 mm entering the drift (12114 out of a total of 16842 individuals) agrees with the hypothesis that the vast majority of drift organisms are in their first stages of development (Waters, 1972). Some studies concluded that the distance travel in the drift depended on the species and on the development stage: juveniles travelled over distances 3 times larger than more mature stages, so their chances to be captured increased 3 times (Allan and Feifarek, 1989). *Baetis muticus, B. rhodani, Epeorus assimilis, Rhithrogena cf carpatoalpina* and *Habroleptoides confusa* were present only in the drift samples with low numbers of individuals, not exceeding 53 (Table 6).

In the samples collected during the day, between 9 a.m. and 6 p.m., the number of mayfly individuals smaller than 2 mm exceeded 80%. Individuals ranging from 2 to 5 mm recorded 11-14%, while individuals larger than 5 mm recorded less than 6%. During the night, a completely different situation was recorded: the percent of small individuals (under 2 mm) ranged between 78% at 9 p.m. and 50% at midnight. The percentage abundance of individuals ranging from 2 to 5 mm varied between 18% at 9 p.m. and 22% at midnight, while larger individuals (>5 mm) recorded 4% at 9 p.m., 27% at midnight and 20% at 3 a.m. Thus, large individuals recorded percentages 3 times greater in some samples collected during the night compared with those taken during daytime (Fig. 7). In the benthos samples 67.5% of mayflies are smaller than 2 mm, 22% of them have body length ranging from 2 to 5 mm and 10 % are larger than 5 mm.

Table 6.

Mayfly taxa and their number of individuals in the drift samples, together with the species densities from the benthos samples, considering the twelve size classes

Taxa	Drift	Size classes												
	Benthos	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Total
Baetis melanonyx	no. ind.	0	0	1301	367	833	1053	461	101	3	0	0	0	4119
	ind./ m^2	0	0	115	19	41	41	44	22	0	0	0	0	281
Baetis muticus	no. ind.	0	0	0	0	3	0	0	0	0	0	0	0	3
	ind./ m^2	0	0	0	0	0	0	0	0	0	0	0	0	0
Baetis rhodani	no. ind.	0	0	17	1	4	7	6	13	5	0	0	0	53
	ind./ m^2	0	0	0	0	0	0	0	0	0	0	0	0	0
Baetis. vernus	no. ind.	0	0	1	6	22	17	9	0	0	0	0	0	55
	ind./ m^2	0	0	0	0	0	0	4	0	0	0	0	0	4
Baetis sp.	no. ind.	4858	7115	63	0	0	0	0	0	0	0	0	0	12036
	ind./ m^2	481	378	0	0	0	0	0	0	0	0	0	0	864
Serratella ignita	no. ind.	22	47	7	1	0	0	0	0	0	0	0	0	77
	ind./ m^2	4	11	7	0	0	0	0	0	0	0	0	0	22
Ecdyonurus sp.	no. ind.	3	9	2	1	3	3	3	1	3	0	0	0	28
	ind./ m^2	0	0	7	0	0	4	0	0	0	0	0	0	11
Epeorus assimilis	no. ind.	0	0	2	0	0	1	0	0	1	0	0	0	4
	ind./ m^2	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhithrogena circumtatrica	no. ind.	0	0	40	10	0	0	1	0	0	0	0	0	51
	ind./ m^2	0	0	33	26	4	0	0	0	0	0	0	0	63
Rhithrogena sp1	no. ind.	0	0	1	2	3	24	73	56	19	4	0	0	182
	ind./ m^2	0	0	0	0	0	4	11	11	0	0	0	0	26
Rhithrogena cf carpatoalpina	no. ind.	0	0	0	0	0	0	0	1	3	0	0	0	4
	ind.mp	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhithrogena sp.	no. ind.	17	43	98	2	0	0	0	0	0	0	1	0	161
	ind./ m^2	33	11	48	0	0	0	0	0	0	0	0	0	92
Habroleptoides confusa	no. ind.	0	0	0	4	2	1	0	0	0	0	0	0	7
	ind./ m^2	0	0	0	0	0	0	0	0	0	0	0	0	0
Subimago	no. ind.	0	0	0	0	0	6	34	13	4	4	0	1	62
	ind./ m^2	0	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations: *ind. = individuals*

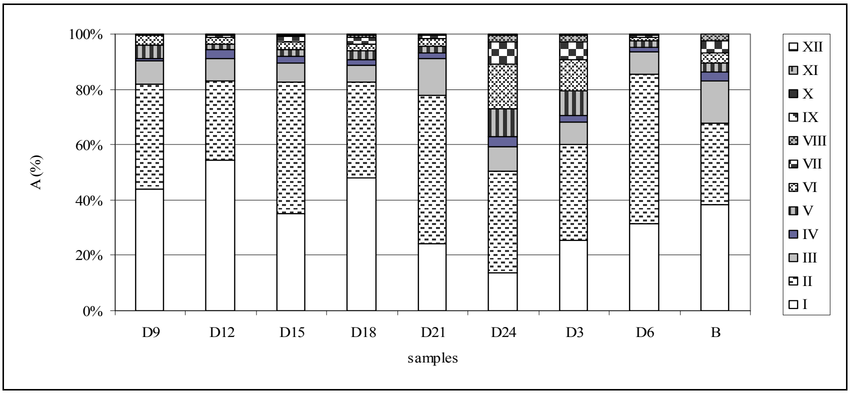

Fig. 7. The percentage abundance (A%) of mayfly individuals of different sizes

Out of the total of 4119 individuals belonging to *Baetis melanonyx* captured in the drift samples, 3583 were collected at night, while only 536 during daytime. Besides the number of specimens, the size of them also increased in the drift starting from 9 p. m. (Fig. 8). Individuals smaller than 3 mm were dominant in the daytime samples, while at midnight and at 3 a.m. individuals ranging from 5 to 6 mm dominated, followed by those with a length of 4-5 mm. Individuals exceeding (exceeding 7 mm) were also well represented.

53 individuals of *Baetis rhodani* were collected; almost half of them (25 specimens) at 3 a.m. (Fig. 8). During the day, the species was present only at noon, with 4 individuals: 3 not exceeding 3 mm and only one over 8 mm. Out of the 49 individuals captured at night, 31 ranged between 4 and 8 mm and 4 individuals exceeded 8 mm.

55 individuals belonging to *Baetis vernus* (Fig. 8) were identified in the drift samples; 15 were found in the daytime samples and 40 were collected at night. This species was absent at 9 a.m.; only one individual was collected at 3 p.m. (smaller than 3 mm) while at 9 p.m. 17 individuals were identified, 14 with a length ranging from 4 to 7 mm.

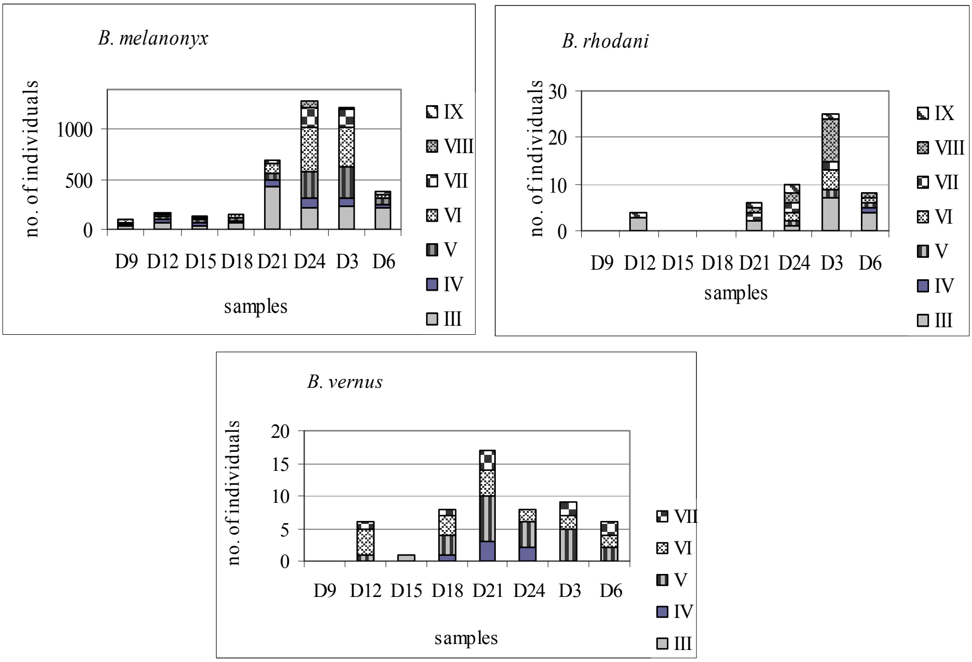

Fig. 8. Size classes of *Baetis melanonyx, B. rhodani and B. vernus* individuals in the drift samples

Even if the number of *Serratella ignita* (Fig. 9) individuals increased during the night, all of them were smaller than 4 mm.

Similarly, the number of *Rhithrogena circumtatrica* specimens (Fig. 9) in the daytime samples was much lower compared to those captured at night. The highest number was recorded at midnight; and the largest individual was collected at 3 a.m. (6-7 mm). This species is cited for the first time in Romania. Up to now it was recorded in Austia, Germany, Poland and Slovenia (Thomas and Belfiore, 2011). The identity of the species was subsequently confirmed by genetic analyses. A fragment of the cytochrome oxidase 1 (cox1) mitochondrial gene was used, and this specimens were in the same cluster than *Rhithrogena circumtatrica* topotype specimens in a European *Rhithrogena* cox1 phylogenetic tree (Vuataz, in prep).

During the day, only 4 individuals belonging to *Rhithrogena* sp1 were caught (3 at noon and 1 at 6 p.m., with sizes ranging between 3 and 7 mm). Out of the 178 individuals collected at night, 99 had a length that ranged between 4 and 7 mm, while 79 exceeded 7 mm. The highest number of individuals captured at night was recorded at 3 a.m., and the lowest number was at 6 a.m., when only one individual was found (Fig. 9).

Ecdyonurus individuals larger than 7 mm were collected only at night, with a maximum number of individuals at 9 p.m. The genus was absent at 9 a.m. and at 6 p.m. (Fig. 9).

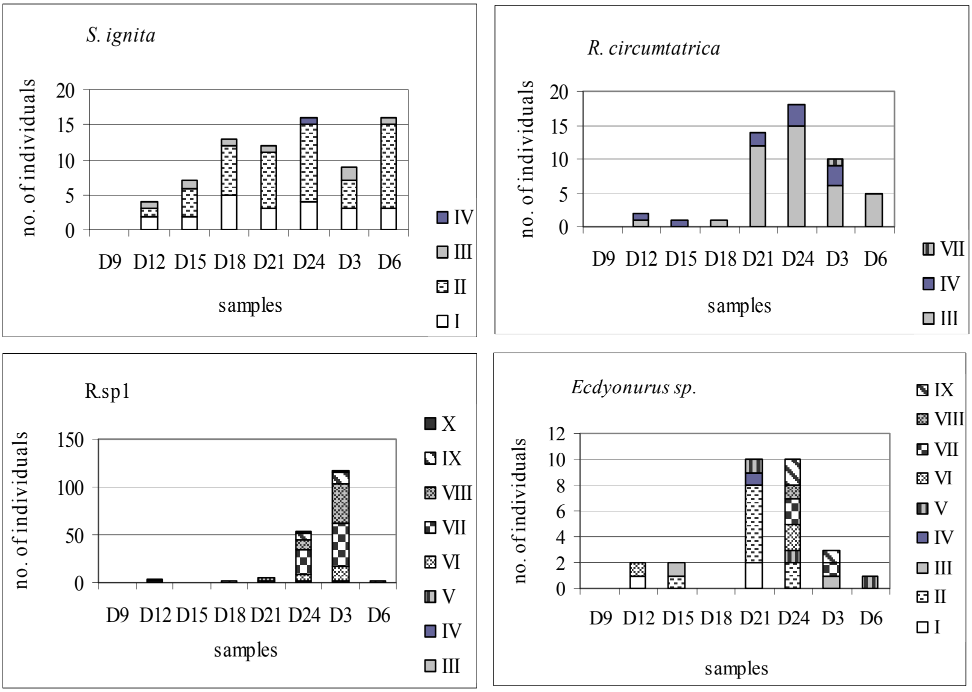

Fig. 9. Size classes of *Serratella ignita, Rhithrogena circumtatrica, R.* sp1 *and Ecdyonurus* sp. individuals in the drift samples

The Principal Component Analysis (PCA) was used to see if the frequency of appearance of mayflies was influenced or not by the time of the sampling. All identified taxa were considered for this analysis, together with their number and the hour of sampling. The first two axes, F1 and F2, explained 70.89% of the total variance. All samples correlated with the first axis F1, except for the sample collected at 9 p.m. (D21). All taxa were distributed on F1 (54.31%), except for *Serattella ignita* and *Rhithrogena cf carpatoalpina,* distributed on F2 (16.58%). Only 4 individuals belonging to *Rhithrogena cf carpatoalpina* were identified in the drift samples, 2 sampled during the day and 2 at night, *Serratella ignita* recorded 77 individuals, 53 captured at night and only 24 during the day. This species' distribution was not well explained by the PCA ordination, probably because only juveniles were found, not exceeding 4 mm. Taxa apearing in the night samples were aggregated in the right side of the graph. Subimagoes, in the left side, are known to enter the drift during the day (Brittain, 1980). The PCA ordination proved that light represented a very important factor influencing the mayfly drift, all taxa recording a strong tendency towards the night drift behavior, except for the subimagoes, that preferred to enter the drift during the day (Fig. 10).

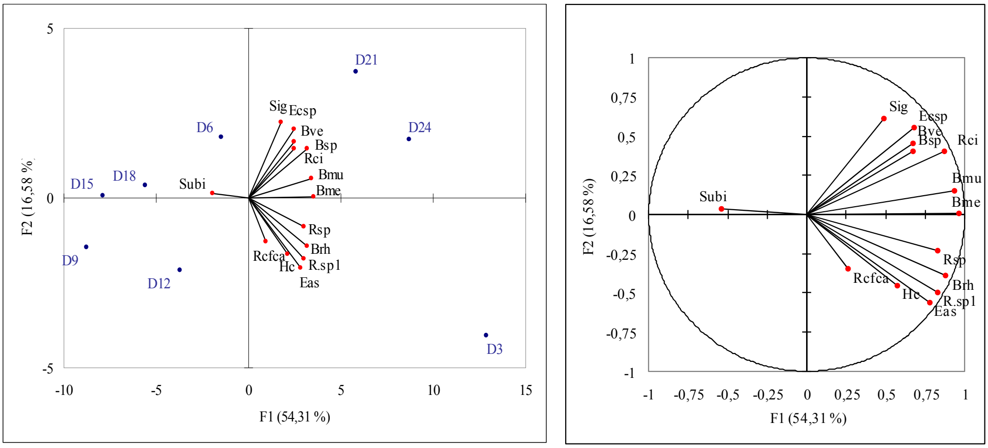

Fig. 10. PCA ordination diagram (Principal Component Analysis) with the mayfly species and the capture times in the drift samples (D9-D6)

The relationship between the physical and chemical parameters, the sampling periods and the size of the mayfly species entering the drift was described by the Canonical Correspondence Analysis (CCA). Size classes for species represented by less than 10 individuals were not considered. The physical and chemical parameters included in CCA were water temperature and the quantity of dissolved oxygen. The first two axis of canonical correlation, F1 and F2, explained 100% the relationship between the species sizes and the environmental factors. The first axis (F1- 93,5%)

represented a gradient of increasing temperature and the second axis (F2 – 6,5%) a gradient of decreasing oxygen (Fig. 11). Two clusters could be distinguished, above and below F1: the first one included the samples collected at night (D21, D24, D3, D6), and the second one the ones taken during the day (D9, D12, D15, D18). Only medium-sized individuals belonging to *Rhithrogena cicumtatrica* were distributed on F2, because they were more abundant in the samples characterized by lower quantities of dissolved oxygen. All other species were distributed on F1. In the first cluster (the samples collected at night), medium-size and large-size individuals were present, except for small mayflies belonging to *Rhithrogena circumtatrica* and *Baetis melanonyx*. The last species had representatives in all samples, but it recorded high numbers at 9 p.m. *Rhithrogena circumtatrica* recorded an atypical drift behavior, probably due to their young stage of development (78% from the larvae did not exceed 3 mm). In the second cluster (the samples collected during daytime), only small individual belonging to *Serratella ignita* and *Ecdyonurus* sp. were present, together with medium-size representatives of *Rhithrogena circumtatrica*.

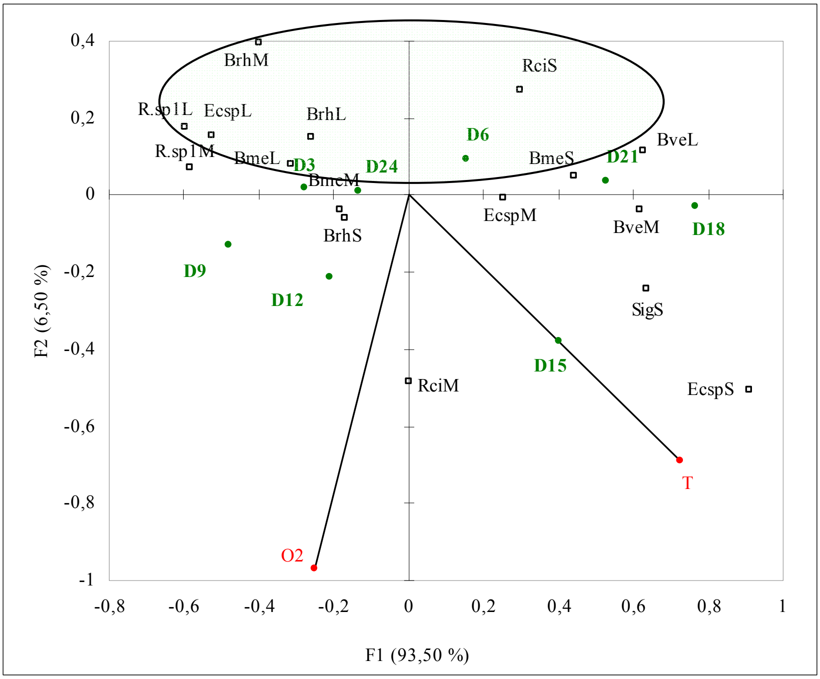

Fig. 11. CCA ordination diagram (Canonical Correspondence Analysis) for the mayfly individuals of different sizes and the environmental factors at the sampling site located downstream the Someşul Cald River gorge: S – small, with a length (L) lower than 3 mm; M – medium (L= 3-6 mm); L – large (L>6 mm); O_2 – dissolved oxygen, T- water temperature

Our results agree with the literature that attempts to explain the adaptative significance of the drift (Elliott, 2002; Holomuzki and Van Loan, 2002) and its role in population mechanisms (Englund and Hambäck, 2004). Our findings support the idea that the night drift is more intense than the day drift.

Passive or active drift includes three activities: entering the water current, transport and attaching to the substratum. The level of control over these processes varies with the taxon, the water velocity, the developmental stage of the individual (Hoover, 2008; Allan and Feifarek, 1989) and the movement ability (Fingerut *et al.*, 2006). Our data showed that *Baetis melanonyx*, *B. vernus, B. rhodani, Rhithrogena* sp1*, Ecdyonurus* sp. had a behavioral drift according to Waters (1965, 1972). Not only the number, but also the size of the individuals captured at night were larger. Young larvae belonging to *Baetis* and *Rhithrogena* genera were present in all drift samples, but their number was again increased during the night.

Observations made on the fish fauna from the upper Someşul Cald River support our findings (Pricope *et al.*, 2009). Several experiments proved that after a first contact with predators, once the nocturnal behavior is established, mayflies avoid entering the drift during the day, even when they return to habitats with no predators (Kohler, 1985; McIntosh and Townsend, 1995; Peckarskz and McIntosh, 1998). *Baetis muticus*, *Epeorus assimilis*, *Rhithrogena cf carpatoalpina* and *Habroleptoides confusa* were represented by no more than 7 individuals in all drift samples, they can be considered species with accidental occurrence. The frequency of this species was below 37% (except for *Habroleptoides confusa* that exceeded 60%) in the drift samples and they were absent from the benthos samples. Their low percentage in the drift samples might be explained if we consider the drift abundance of macroinvertebrates to be directed proportional to their abundance in regular benthos samples (Statzner *et al.*, 1984; Ramirez and Pringle, 1998). For *Serratella ignita* and *Rhithrogena circumtatrica* even if a tendency of increasing in numbers during the night was observed, it was not followed by increases in size. However, individuals collected from the benthos samples were also small, so drift individuals were analogous considering the development stage.

Conclusions

Mayflies represented the dominant group in the drift samples. From the total of 27399 individuals collected in the drift samples, mayflies represented 61.5%. They also recorded 49.5% from the total density of benthic organisms captured in the benthos samples, which reached 2751.96 individuals/m^2.

Mayflies recorded highest numbers during the night. They were followed by chironomids and stoneflies (Plecoptera) that reached maximum numbers during the day.

Our data showed that *Baetis melanonyx, B. vernus, B. rhodani, Rhithrogena* sp1*, Ecdyonurus* sp. had a behavioral drift: not only the number, but also the size of the individuals entering the drift were larger at night.

Even if the abundance of *Rhithrogena circumtatrica* and *Serratella ignita specimens* was higher during the night, their size proved to be smaller, due to their developmental stage at the time of sampling.

Baetis muticus, Epeorus assimilis, Rhithrogena cf carpatoalpina and *Habroleptoides confusa* were absent from the benthos samples and recorded low numbers in the drift samples.

The importance of light in mayflies drift is sustained by results of PCA and CCA ordination.

Rhithrogena circumtatrica is cited for the first time in Romania in the present paper.

Acknowledgments

Thanks are given to my colleagues Laura Momeu, PhD for her support; to Karina Battes, PhD and Mugur Bogătean for their help in collecting the samples and measuring the physical and chemical parameters. I also recognize with gratitude the help of Professor Michel Sartori, PhD, for his taxonomical expertise and to PhD student Laurent Vuataz for providing the results of genetic analyses.

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NEW DATA CONCERNING THE BENTHIC DIATOM COMMUNITIES FROM THE ȚIGANILOR RIVULET (“ALEXANDRU BORZA” BOTANICAL GARDEN, CLUJ – NAPOCA, ROMANIA)

ADRIANA OLENICI[1,✉] and LAURA MOMEU[1]

SUMMARY. This work presents the results of the studies on the benthic diatom communities from the Țiganilor Rivulet (“Alexandru Borza” Botanical Garden, Cluj - Napoca, Romania) in 2008. Comparing our data with that from two similar studies, one carried out in 1999 by Morari (Morari, 2000), the other one in 2001 by Neag *et al., (*2005), we set the following objectives: to establish the qualitative composition of the benthic diatom communities, to conduct quantitative evaluations, to observe the seasonal and multiannual dynamics and to evaluate the water quality based on the indicator diatom species. From the fourteen samples collected during 2008, we identified only diatom species (*Bacillariophyta*). We found 63 taxa, belonging to 18 genera, the best represented being *Navicula* and *Nitzschia.* From the qualitative structure point of view, the number of diatom species varied according to the season: 28 species in spring, 29 species in summer and 48 species in autumn. Quantitatively, in the samples collected in spring, the species *Navicula gregaria* prevailed, followed by *Nitzschia palea.* In summer, the most common species was *Diadesmis contenta,* followed by *Navicula gregaria*, *Nitzschia palea*, *N. constricta* and *N. intermedia.* In autumn, the predominant species was *Navicula gregaria,* followed by *Nitzschia palea*, *N. intermedia* and *Navicula mutica.* The analysis of the diatom community structure showed a seasonal dynamics, with a peak of development in autumn. The floristic similarity had relatively low values. In addition, the Canonical Correspondence Analysis (CCA), the equitability and the Shannon’s diversity suggested, once again, the seasonal dynamics. Considering the saprobity, apart from the indifferent species, more than half (57%) of the diatoms identified indicated a critical saprobic level. Many of the species were also indicators of eutrophic waters, according to the salinity and conductivity values. The comparative study was based on the qualitative composition of the diatom communities from 1999, 2001 and 2008. The values of floristic similarity were low, suggesting a multiannual dynamics. Strong human impacts, like accidental sewage evacuations, could be the cause of this particular situation.

Keywords: diatom, seasonal dynamics, trophicity, saprobity, indicator species.

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Introduction

Diatoms are a very distinct group of algae, identifiable under the light microscope by their yellow - brown coloration and by the presence of a thick silica cell wall (Bellinger and Sigee, 2010). The potential for freshwater organisms to reflect changes in environmental conditions was first noted by Kolenati (1848) and Cohn (1853), who observed that biota in polluted waters were different from those in non - polluted situations (Liebmann, 1962). Two major arguments in favor of using diatoms in water - quality assessments are that their distributions are cosmopolitan and their ecology is well studied. If these assumptions are true, diatom - based monitoring tools could be considered universal and used in any geographic area (Potapova and Charles, 2005). Diatoms are widely used to monitor river pollution because they are sensitive to water chemistry, especially to ionic content, pH, dissolved organic matter and nutrients. Wide geographic distribution and well studied ecology of most diatom species are cited as major advantages of using diatoms as indicator organisms (McCormick and Cairns, 1994). At the same time water quality has begun to deteriorate increasingly, mainly as a result of the physical, chemical and bacteriological alterations, and the aquatic ecosystems are evermore affected by various types of pollution, the anthropic one being almost always included. A good example is the Țiganilor Rivulet, which has suffered along the years from the accidental sewage evacuations (Neag *et al.*, 2005). The short and relatively poor course of this rivulet divides the “Alexandru Borza” Botanical Garden (Cluj - Napoca, Romania) into two almost equal parts, furrowed by three tributaries, thus adding the picturesque charm of this place (Pop, 1966). An important stimulus for our study was obtaining the data and using it together with the information retrieved from 1999 (Morari, 2000) and 2001 (Neag *et al.*, 2005), to offer an overview on the dynamics of the algal communities. Therefore, our objectives were: to establish the qualitative composition of the benthic diatom communities, to conduct quantitative evaluations, to observe the seasonal and multiannual dynamics and to evaluate the water quality based on the indicator diatom species. In this context, this study is in the same time an addendum as well as an extension of the previous studies.

Materials and methods

The samples (14) were collected from one sampling site from different substrata, in spring (S1 - S5), summer (S6 - S9) and autumn (S10 - S14) of 2008, using different methods depending on the substratum type (stones, fine sediment, aquatic macrophytes, wooden surface) (Barber and Haworth, 1981). This sampling site is the same that was chosen for the studies from 1999 (Morari, 2000) and 2001 (Neag *et al.*, 2005) (Fig. 1). A series of physical and chemical parameters have been measured, using the portable meters: YSI Model 52 oxygen meter, CONSORT Model P902 pH meter, CONSORT Model K119 conductivity and salinity meter and thermometer. The samples were preserved in the field with 4% formaldehyde. Species identification was carried out using a Nikon Eclipse E400 microscope, according to usual diatom keys (Krammer, 2002, Krammer and Lange - Bertalot, 1986, 1988, 1991 a, b). The quantitative composition was determined by counting 400 individuals from every sample (Prygiel and Coste, 2000). In the same time we calculated the species equitability (*E*) and the Shannon's diversity (*H*), using the relative abundance data (Shannon, 1948, Washington, 1984). In order to calculate the Shannon index, the following formula was used: $H = -\sum_{i=s}^{s} p_i \ln p_i$, where s = the number of species in a comunity and p_i = the share of each species in a comnunity; the formula used for determining the equitability was: $E = \frac{H}{\ln S}$, where lnS = H_{max}. The analysis of the canonical correspondence (CCA) between the species, the samples and the values of the physical and chemical parameters, was realized using the XLSTAT software (Addinsoft, 2009). The floristic similarity between the samples was calculated with the PAST program (Hammer *et al.*, 2001), using the Jaccard index (Jaccard, 1901): Ij = c / (a + b + c), where: a = the number of species in sample a; b = the number of species in sample b; and c = the number of species common to samples a and b.

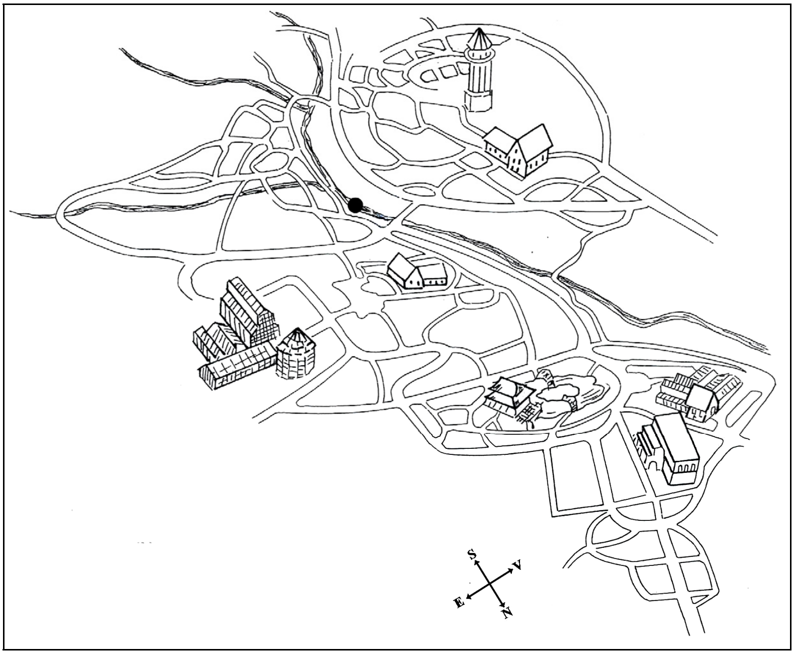

Fig. 1. The sampling site from the Țiganilor Rivulet ("Alexandru Borza" Botanical Garden, Cluj – Napoca, Romania) (changed after Micle *et al.*, 2002)

Results and discussions

Influence of physical and chemical parameters on diatom communities

The pH was fluctuating within pH range 7.10 and 8.30; the oxygen levels, the atmospheric and water temperatures differed according to the season. The conductivity values suggested a high concentration of inorganic chemical substances in the water in March (2008), compared to August and October (2008), values that followed closely those of the water salinity (Table 1).

Table 1.

The values of the physical and chemical parameters measured in 2008

Parameters-Value/m.u.	***March***	***August***	***October***
pH	8.32	7.80	7.10
O_2 (%)	82	13	77
Atmospheric temperature (°C)	12.0	26.0	11.2
Water temperature (°C)	8	17	9
Conductivity (µS/cm)	597	203	107
Salinity (mg/l)	-	109	201

The CCA ordination diagram shows the patterns of variation in community’s composition that can be explained best by the environmental variables and also visualizes approximately the “centers” of the species distributions along each of the environmental variables. Such a diagram effectively summarizes relationships between the communities and environment for data sets on algae along the gradients (Ter Braak, 1986, 1987, Legendre and Legendre, 1998, Greenacre, 2007). The algal communities identified from the samples S1 to S5 (March) showed a positive correlation with the conductivity, the pH and dissolved oxygen in the water, and a negative correlation with the water temperature. This suggested the presence of the species that preferred slightly alkaline and colder waters and also tolerated a high content of dissolved oxygen and inorganic chemical substances. A positive correlation was found between the diatom communities identified in the sample S6 (August) and the water temperature. At the same time, these communities had a negative relationship with the oxygen (Fig. 2). We can conclude that these species tolerated waters with low quantities of oxygen and high temperatures. The communities from the samples S11, S12 and S13 (October) were negatively correlated with water pH and conductivity. This finding suggested the presence of species preferring waters with a low content of inorganic chemical substances and oxygen. Among the dominant species, *Navicula gregaria* and *Nitzschia palea* tolerated alkaline waters, with a high level of dissolved oxygen and inorganic chemical substances. *Diadesmis contenta* preferred warm, less oxygenated waters, and *Navicula mutica* and *Nitzschia intermedia* were found in waters with a low content of inorganic chemical substances and a neutral pH (Krammer, 2002, Krammer and Lange - Bertalot, 1986, 1991 b).

At the same time, we can observe that diatom communities aggregated according to the season, as expected (Fig. 2).

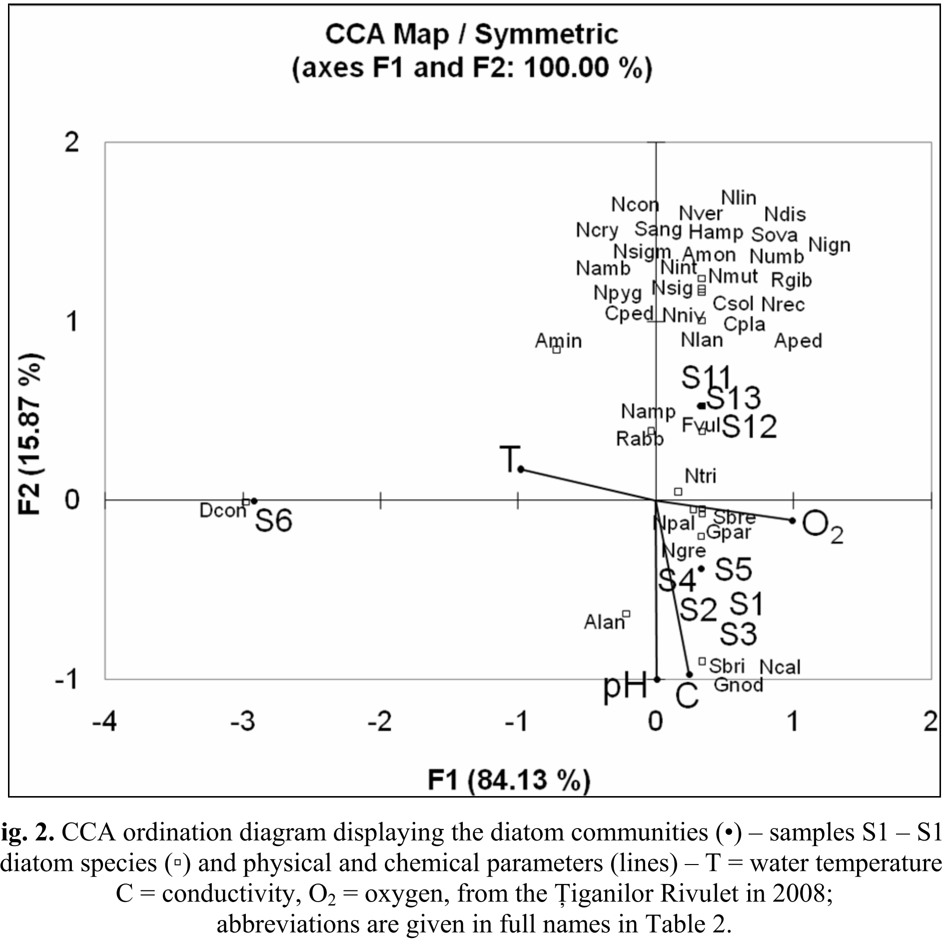

Fig. 2. CCA ordination diagram displaying the diatom communities (•) – samples S1 – S13, diatom species (▫) and physical and chemical parameters (lines) – T = water temperature, C = conductivity, O_2 = oxygen, from the Țiganilor Rivulet in 2008; abbreviations are given in full names in Table 2.

The qualitative structure of the diatom communities

In the 14 samples we identified only diatom species. Table 2 presents the 63 diatom taxa found.

Table 2.

Diatom species identified in the Ţiganilor Rivulet in 2008

Taxa	**Abbrevations**	**Samples**													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Achnantes lanceolata	Alan	–	+	–	–	–	+	+	+	–	–	+	+	+	–
Achnantes minutissima	Amin	–	–	+	–	–	+	+	+	–	–	+	+	+	–
Achnantes minutissima var. *saprophila*	Amins	–	–	–	–	–	+	–	–	–	+	+	–	–	+
Amphora montana	Amon	–	–	–	–	–	–	–	+	–	–	–	+	+	–
Amphora pediculus	Aped	–	–	+	–	–	+	–	+	–	–	+	+	–	+
Cocconeis pediculus	Cped	–	–	–	–	–	–	+	–	–	–	+	–	+	–
Cocconeis placentula	Cpla	–	–	–	–	–	+	–	+	–	–	+	+	+	+
Cymatopleura solea	Csol	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Cymbella affinis	Caff	–	–	–	–	–	–	–	–	–	–	+	+	–	–
Cymbella amphicephala	Camp	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Cymbella minuta	Cmin	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Cymbella turgidula	Ctur	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Diadesmis contenta	Dcon	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Diploneis elliptica	Dell	–	–	–	–	+	–	–	–	–	–	–	–	–	–
Epithemia adnata	Eadn	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Epithemia goeppertiana	Egoe	–	–	–	–	+	–	–	–	–	–	–	–	–	–
Fragilaria leptostauron	Flep	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Fragilaria ulna	Fuln	–	–	–	–	–	–	–	–	–	–	+	+	+	–
Frustulia vulgaris	Fvul	–	+	–	–	–	–	–	+	–	–	–	+	+	–
Gomphonema augur	Gaug	–	–	–	–	–	–	–	+	–	–	–	–	–	–

Table 2 (continued)

Taxa	Abbrevations	Samples													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gomphonema gracile	Ggra	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Gomphonema olivaceum	Goli	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Gomphonema parvulum	Gpar	+	+	+	–	+	+	–	–	–	+	+	+	+	+
Gomphonema pumilum	Gpum	–	–	–	–	–	–	–	–	+	–	–	–	–	–
Gyrosigma nodiferum	Gnod	–	–	–	+	–	–	–	+	–	–	–	–	–	–
Gyrosigma scalproides	Gsca	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Hantzschia amphioxys	Hamp	–	+	–	+	–	–	+	–	–	–	+	+	+	–
Navicula ambigua	Namb	–	–	–	+	–	–	–	–	–	–	+	–	–	–
Navicula cincta	Ncin	+	+	–	–	–	–	–	–	–	–	–	–	+	–
Navicula cryptocephala	Ncry	–	–	–	–	–	–	+	–	–	–	+	–	+	–
Navicula gallica	Ngal	–	–	–	–	–	+	–	–	–	–	–	–	–	–
Navicula goeppertiana	Ngoe	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Navicula gregaria	Ngre	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Navicula ignota	Nign	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Navicula kotschyi	Nkot	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Navicula lanceolata	Nlan	+	+	+	–	+	–	–	–	–	–	–	+	+	–
Navicula mutica	Nmut	–	+	–	–	–	+	–	+	–	–	+	+	+	–
Navicula mutica var. *ventricosa*	Nmutv	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Navicula nivalis	Nniv	–	–	–	–	–	–	–	–	–	–	+	+	+	–
Navicula pygmaea	Npyg	–	–	–	–	–	–	+	+	–	–	+	+	+	–
Navicula recens	Nrec	–	–	–	–	–	–	–	–	–	–	+	–	+	–
Navicula tripunctata	Ntri	+	+	+	–	+	+	+	–	–	–	+	+	+	–

Table 2 (continued)

Taxa	Abbrevations	Samples													
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Navicula ventricosa	Nven	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitzschia amphibia	Namp	–	–	–	+	–	+	+	–	–	–	+	+	–	–
Nitzschia calida	Ncal	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Nitzschia constricta	Ncon	–	–	–	+	–	–	–	–	+	+	+	+	+	+
Nitzschia debilis	Ndeb	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitzschia dissipata	Ndis	–	–	–	–	–	–	–	–	–	+	+	+	+	–
Nitzschia intermedia	Nint	–	–	–	+	–	–	+	+	+	+	+	+	+	+
Nitzschia linearis	Nlin	–	–	–	–	–	–	–	–	–	–	+	+	+	–
Nitzschia palea	Npal	+	–	+	+	+	–	+	+	–	+	+	+	+	+
Nitzschia sigma	Nsig	–	–	–	–	–	–	–	–	–	+	+	+	–	+
Nitzschia sigmoidea	Nsigm	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitzschia tryblionella	Ntry	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Nitzschia umbonata	Numb	–	–	–	–	–	–	–	+	+	+	+	+	+	–
Nitzschia vermicularis	Never	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Rhoicosphenia abbreviata	Rabb	+	+	+	–	+	+	+	+	–	–	+	+	+	+
Rhopalodia gibberula	Rgib	–	–	–	–	–	–	–	–	–	–	+	–	–	–
Surirella angusta	Sang	–	–	–	–	–	–	–	–	–	–	+	+	+	–
Surirella brebissonii	Sbre	–	+	+	+	+	–	–	+	–	–	+	+	+	+
Surirella brightwillii	Sbri	–	–	–	+	–	–	–	–	–	–	–	+	–	–
Surirella ovalis	Sova	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Surirella terricola	Ster	–	+	–	–	–	–	–	–	–	–	–	–	–	–

It was observed that *Navicula* and *Nitzschia* genera dominated all diatom samples from all seasons (Fig. 3, 4, 5).

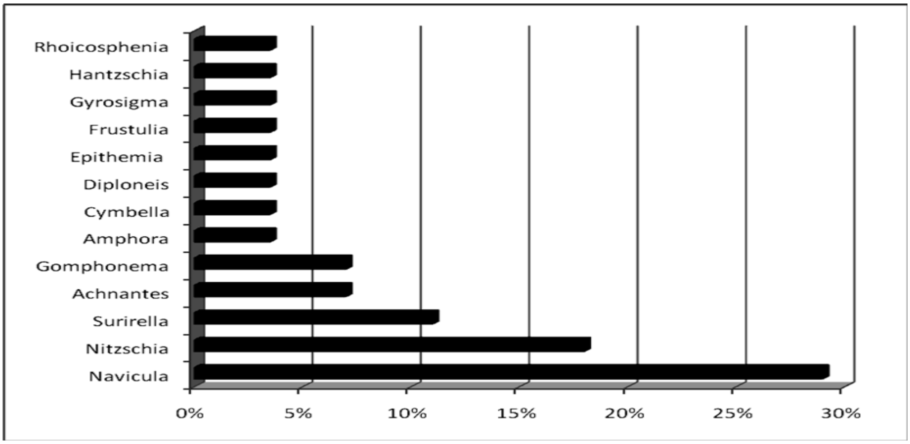

Fig. 3. The estimated percentage of the diatom species within the genera in samples collected from Țiganilor Rivulet (S1 – S5) during spring time

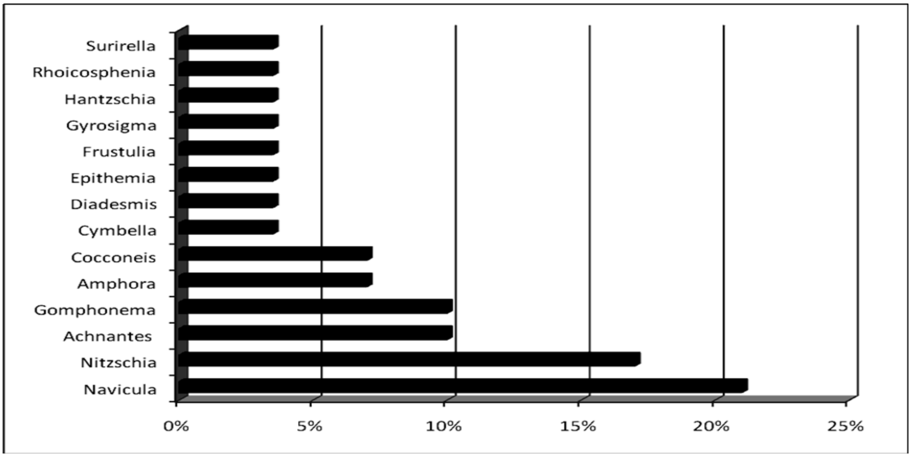

Fig. 4. The estimated percentage of the diatom species within the genera in samples collected from Țiganilor Rivulet (S6 – S9) during summer time

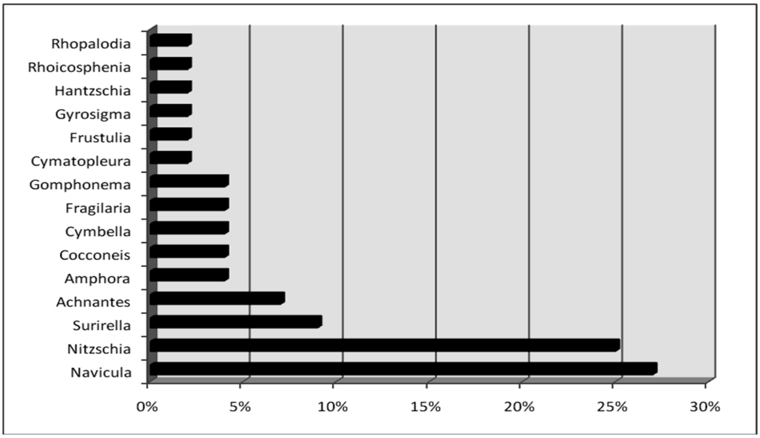

Fig. 5. The estimated percentage of the diatom species within the genera in samples collected from Țiganilor Rivulet (S10 – S14) during autumn time

Aspects concerning the quantitative structure of diatom communities

Quantitative analyses were possible in 9 out of the 14 samples. Accordingly, in 4 of the spring samples the dominating species was *Navicula gregaria* (87.35% - 99.78%) and in another one *Nitzschia palea* (81.62%). In a summer sample *Diadesmis contenta* (95.75%) was the most common, in 3 of the autumn samples the most common ones were *Navicula gregaria* (34.50% - 63.53%), *Nitzschia palea* (10.37% - 25.75%) and *Nitzschia intermedia* (9.93% - 26.75%) and in another one also *Navicula mutica* (11.24%) (Fig. 6). In the samples where the analysis of the

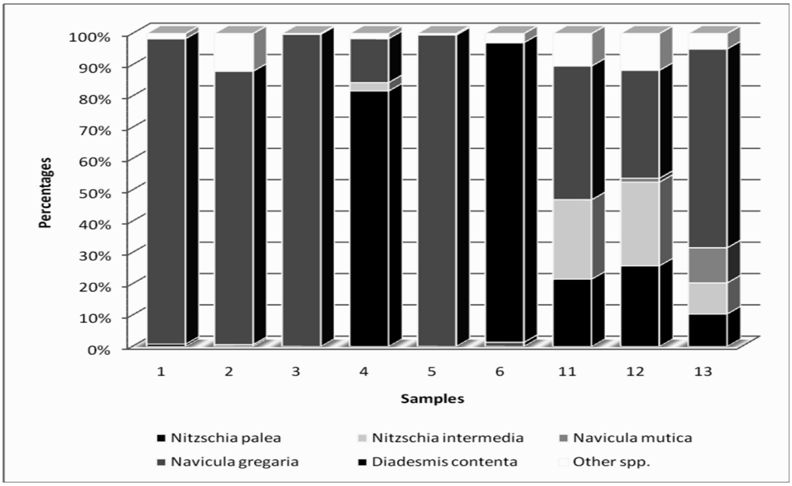

Fig. 6. The quantitative percentages of the diatom species in the samples collected from the Țiganilor Rivulet in 2008

quantitative structure was not possible, we observed that *Navicula gregaria*, *Nitzschia constricta*, *Nitzschia intermedia and Nitzschia palea* were dominant.

The highest values of the number of species, equitability and Shannon's diversity were recorded by diatom communities from samples 11 and 12 (autumn), and the lowest by those from the samples 3 and 5 (spring) (Fig. 7). This may be due, partially, to the quantity of nutrients and organic matter present in the water when the samples were collected.

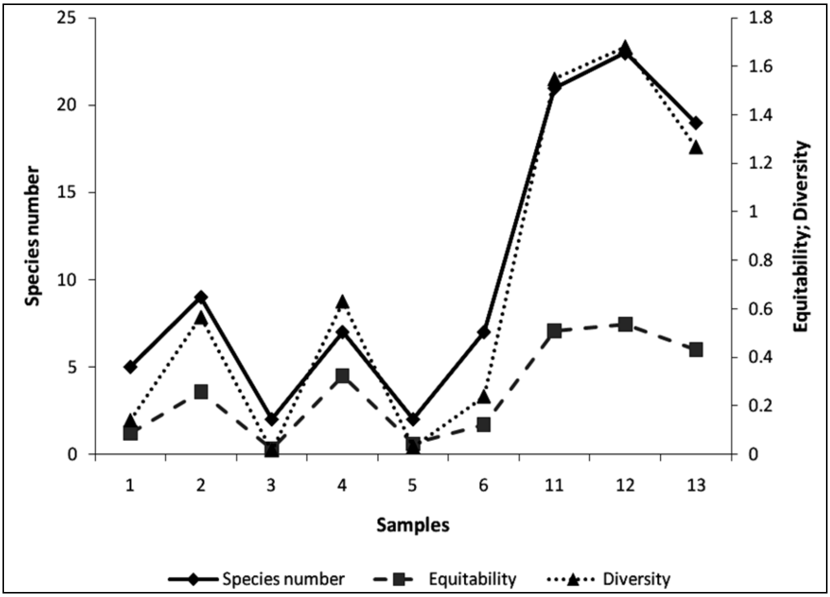

Fig. 7. The variation of the species number, equitability and Shannon's divesity in the Țiganilor Rivulet (2008)

The seasonal dynamics of the diatom communities

The samples were collected in March, August and October, in order to observe the seasonal dynamics of the algal communities. Qualitatively, the number of diatom species varied according to the season. 28 species were identified in spring, 29 in summer and 48 in autumn. From the quantitative point of view, *Navicula gregaria* and *Nitzschia palea* were the dominant species during all three seasons. These results are partially in agreement with those from the work of Patrick (1977), according to which diatoms have two development peaks, one in the spring and one in the autumn. In our case the maximum development was detected in autumn. Moreover, the CCA, equitability and Shannon's diversity suggested a seasonal dynamics (Fig. 2 and 7).

Floristic similarity

Using the presence - absence data of the samples in all three seasons, we constructed a dendrogram. This dendrogram showed the relatively low levels of floristic similarity between aggregates which grouped together communities from different seasons, suggesting once again a seasonal dynamics. The highest values of the Jaccard index were observed between the communities sampled in the same season. Thus, three aggregates were formed: S3, S5, S1, and S2 that corresponded to spring samples, S6, S7 and S8 to summer ones and S11, S12, S13 and S10, S14 to autumn ones, suggesting a tendency of the diatom communities towards a seasonal grouping (Fig. 8).

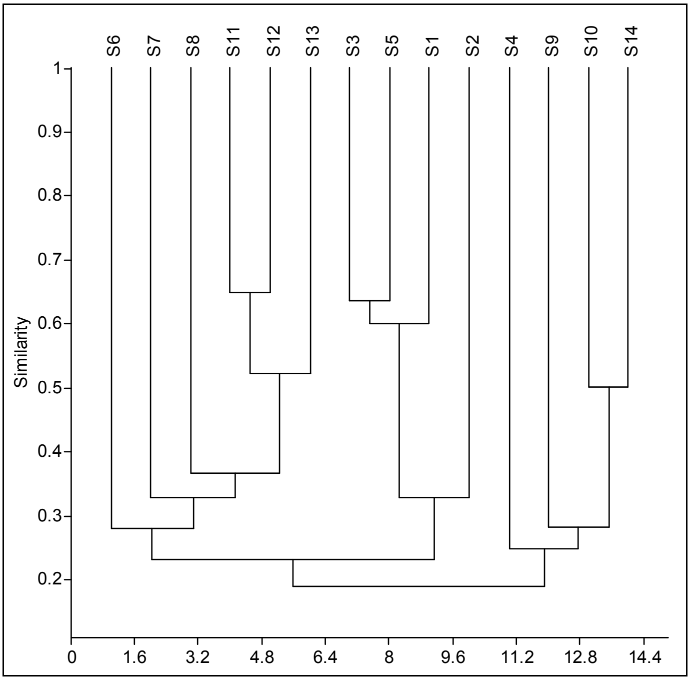

Fig. 8. The floristic affinity relationships between diatom communities sampled from the Ţiganilor Rivulet in 2008: March (S1 - S5), August (S6 - S9) and October (S10 - S14)

The trophicity level of the water

Some of the identified species preferred a low level of trophicity (oligotrophic: *Cymbella amphicephala, Cymbella minuta*), but most of them a medium to high level (mesotrophic: *Navicula recens*, *Rhoicosphenia abbreviata*; eutrophic: *Navicula pygmaea, Nitzschia umbonata*), one of them actually being hypertrophic (*Nitzschia palea*). Some of dominating species were characteristic to eutrophic waters, rich in electrolytes, too. These showed a high trophicity in the Ţiganilor Rivulet in 2008.

The saprobity level of the water based on the indicator diatom species

Out of the 63 diatom species from the Țiganilor Rivulet, 46 showed different values of saprobity, as follows: 2 species were oligosaprobic, 4 species oligoβ - mesosaprobic, 14 species β - mesosaprobic, 10 species βα - mesosaprobic, 13 species α - mesosaprobic and 3 species polisaprobic (Rott *et al.,* 1997). From here we concluded that 43% from species indicated a saprobity level varying from oligosaprobic to β - mesosaprobic and 57% one from βα - mesosaprobic to polisaprobic. The dominating species, *Navicula gregaria, Nitzschia constricta* and *Nitzschia palea,* suggested critical saprobic levels. These results confirmed that the elements that tolerate the critical saprobic level were present in a higher percentage than those that indicated a relatively low content of organic matter in the water (Fig. 9). This indicated a high quantity of organic matter in Țiganilor Rivulet in 2008, whitch could be a sign of a mediocre water quality (Zelinka and Marvan, 1961, Sládeček, 1973, 1986).

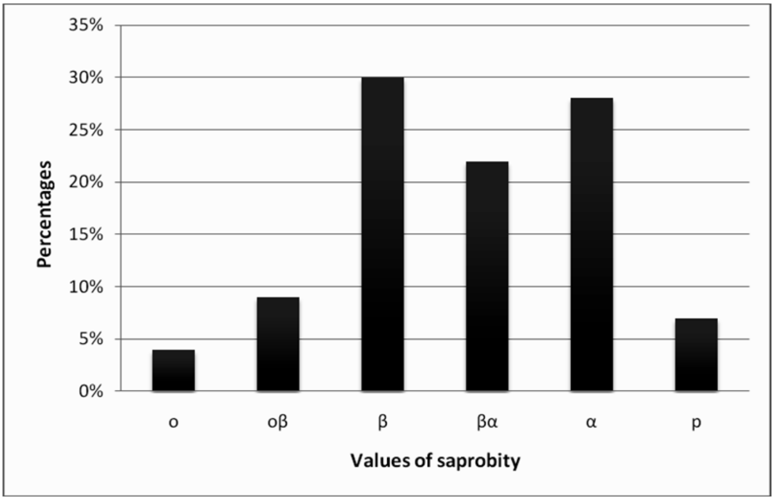

Fig. 9. The percentage of saprobity indicator diatom species from all species identified in the Țiganilor Rivulet in 2008 (o = oligosaprobic, oβ = oligoβ - mesosaprobic, β = β - mesosaprobic, βα = βα - mesosaprobic, α = α - mesosaprobic, p = polisaprobic)

Our results are similar with those of Kozma *et al., (*2001) in a study from Turului Gorge (Transylvania, Romania) for the diatom communities in the Racilor Stream. They are also similar with those acquired by Bíró- Halmágyi *et al., (*2004) in a work in Turda Gorge (Transylvania, Romania), for the diatom communities in the Hăşdate Stream. In this context, the floristic composition of the diatom communities found in the Țiganilor Rivulet is similar to those from the streams analyzed in these two studies. Our results are also in accordance with the results of these two studies, concerning the seasonal dynamics and the use of diatoms in determining the ecological status of water.

Comparative aspects on the qualitative structure of diatom communities from the Ţiganilor Rivulet in different years

The qualitative structure assessment was made by comparing the situation in 2008 to that in 1999 (Morari, 2000) and 2001 (Neag *et al.*, 2005). During 2008 only diatoms were present, while 29 species of algae belonging to *Bacillariophyta* (25 species – 86.21%) and *Chlorophyta* (4 species – 13.79%) were identified in the samples collected during autumn 1999. Of all algal species identified, the diatoms were dominant. The low number of algal species was explained, on one hand, by the fact that only autumn samples were analyzed. On the other hand, this decreased number was accounted by the large quantity of organic matter present in the Ţiganilor Rivulet, from sewer waters, caused by repairs in the sewers system (Morari, 2000). In the samples collected in 2001, in 3 seasons, 81 species were identified: *Bacillariophyta* (69 species – 85.19%), *Chlorophyta* (5 species – 6.17%), *Cyanoprokaryota* (3 species – 3.70%), *Euglenophyta* (4 species – 4.94%) (Neag *et al.*, 2005), the diatoms being, once again, dominant (Fig. 10).

In 2008 we found 10 of the species identified in 1999, 18 from 2001, while 4 species were common in all considered years. The presence of algae belonging to other phyla in 1999 and 2001 indicated a change of the physical and chemical conditions and suggested a multiannual dynamics.

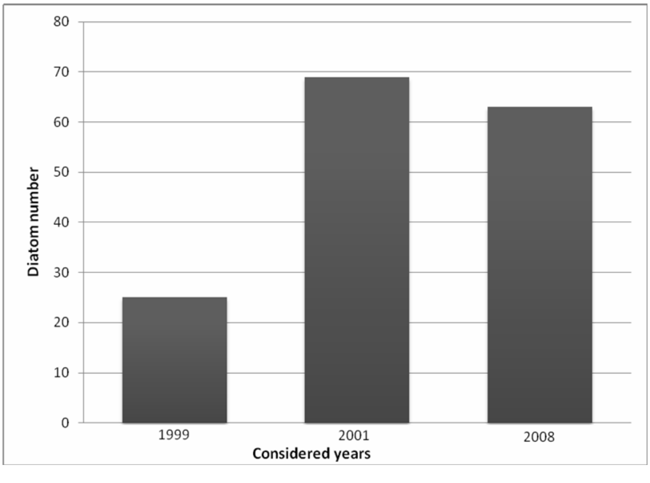

Fig. 10. The number of diatom species identified in 1999, 2001 and 2008, in the Ţiganilor Rivulet

The analysis of the floristic similarity of the diatom communities in these 3 years reveals the association at a level of similarity of 17% between those from 2001 and 2008, and of 15% between these and those from 1999. These very low values indicated once again the multiannual dynamics.

Conclusions

We draw the following conclusions from the study of diatom communities from the Țiganilor Rivulet - the "Alexandru Borza" Botanical Garden (Cluj – Napoca, Romania):

1. In 2008 we found 63 diatom species, belonging to 18 genera, the best represented ones being *Navicula* and *Nitzschia;*
2. The number of diatom species varied with the season (28 species in spring, 29 species in summer and 48 species in autumn);
3. In the spring samples, the dominating species were: *Navicula gregaria* and *Nitzschia palea;* in summer: *Diadesmis contenta*, *Navicula gregaria*, *Nitzschia palea*, *Nitzschia constricta* and *Nitzschia intermedia;* and in autumn: *Navicula gregaria, Navicula mutica, Nitzschia palea* and *Nitzschia intermedia* again;
4. The floristic affinity showed a tendency of the diatom communities towards a seasonal grouping;
5. The floristic similarity, the CCA and the values of equitability and Shannon's diversity suggested the seasonal dynamics of the diatom communities.
6. 17 species were indifferent in terms of saprobity, the others being divided as follows: 43% indicated a saprobic level ranging from low to medium and 57% a critical one. Moreover, many species were indicators of a high level of trophicity;
7. The comparative study of the qualitative composition showed a multiannual dynamics, suggested by the low floristic similarity between the diatom communities pertaining to the considered years and the presence of algae belonging to different phyla.
8. We believe that the main cause of these differences in compositional and structural attributes of the diatom communities of Țiganilor Rivulet was the human impact, manifested through the accidental evacuations of sewage waters.

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THE USE OF PLANKTON COMMUNITY FOR ASSESSING THE ECOLOGICAL STATUS OF LAKE ŞTIUCII, NATURE RESERVE (TRANSYLVANIA, ROMANIA)

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SUMMARY. The assessment of the ecological status of water bodies using biotic communities represents an important and actual issue, according to the Water Framework Directive 2000/60/EC of the European Parliament. Phytoplankton community is recognized as a powerful tool in assessing water quality of lakes, while zooplankton is only considered in isolated cases of eutrophication or accidental pollution. The present paper aims to attain a complete and comprehensive characterization of the ecological status of Lake Ştiucii, using both phytoplankton and zooplankton communities. In case of phytoplankton, several trophic and organic pollution indices were calculated based on the number of phytoplankton species present in the water column. The values of the indices calculated for the years 1943, 1958, 1998, 1999, 2000 and 2005 were compared. The lake evolved from an oligo-mesotrophic state and the lack of organic pollution in 1943/1958, to meso-eutrophic conditions and moderate organic pollution after 1998. Three indices based on planktonic microcrustacean community were considered. Similar to the phytoplankton data, these indices based on zooplankton showed a moderate ecological status for Lake Ştiucii.

Keywords: biotic index, microcrustaceans, organic pollution, phytoplankton, trophic state.

Introduction

The open-water zone in lentic ecosystems includes diverse communities: the organisms suspended in the water (plankton), the active swimmers (nekton) and those that live at the air–water interface (neuston) (Lampert and Sommer, 2007). The classical view of the standing water ecosystems assumes that phytoplankton includes most of the primary producers, that are consumed by zooplankton and that zooplankton are consumed in turn by fishes (Dodds, 2002). Because of their central positions in this simple model of lake ecosystem, phytoplankton and zooplankton are vital components of freshwater aquatic food webs, responding quickly to any changes in the status of their environment, natural or human-induced (Suthers and Rissik, 2009).

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The current European legislation in the field of water policy – the Water Framework Directive 2000/60/EC of the European Parliament and of the Council stipulates the maintaining and improving of the water quality. One of the biological elements chosen for the assessment of surface water status in lakes, transitional and coastal waters is the composition, abundance and biomass of phytoplankton. As for zooplankton, it is not considered to be a key biological element, but it is used in acquiring useful information in case of eutrophication or accidental pollution (according to Order 31/2006).

Lake Ştiucii (N: 46.9676; E: 23.9015) is located at 274.5 m altitude, on the Bonț River valley, a tributary of the Fizeş River. It represents a natural lake with a mean depth of 3.12 m and a total area of 57.35 ha (Şerban and Sorocovschi, 2003). The water source of the lake is represented mainly by superficial runoff, while the groundwater source can become significant during summer or autumn (Sorocovschi *et al.*, 2000). Human impacts are not severe in Lake Ştiucii; they are mostly represented by runoffs from the agricultural and pasture fields near the lake, tourism and fish stocking.

The algal studies in Lake Ştiucii started in 1943, with the first list of algae from this lake, including 13 taxa (Péterfi *et al.*, 1960). 45 species were identified in 1958 (Péterfi *et al.*, 1960). Another study dealing with the primary productivity in several lakes from the Transylvanian Plateau included data concerning the main taxonomic groups of algae from Lake Ştiucii, from 1974 to 1976 (Péterfi *et al.*, 1981). A complex research on phytoplankton, periphyton and primary productivity in Lake Ştiucii started in the 90's and continued in the years 2000 (Momeu *et al.*, 1999-2000; Gudasz *et al.*, 2000; Momeu *et al.*, 2004; Gudasz, 2004). On the other hand, zooplankton from Lake Ştiucii was not so well studied. No data were found in the old works of Daday, or in the chorology synthesis published by Negrea (1962). The first data on planktonic microcrustaceans from Lake Ştiucii were included in two master thesis defended at the "Babeş-Bolyai" University, Cluj-Napoca (Echim, 2000; Mara, 2000). A review of the common microcrustacean species was carried out by Gudasz (2004) for a study period that stretched from January 2001 to December 2002.

The present paper represents an attempt to assess the ecological status of a natural lake using both phytoplankton community and the planktonic microcrustaceans. Including zooplankton in water quality studies generates a complete image on the status of the ecosystem, because it represents an important link in lentic food chains. An evolution of the lake's trophic state and organic pollution load is described, based on planktonic algae indices calculated for different periods of time.

Material and Methods

Phytoplankton data were collected from different studies carried out in 1943, 1958 (Péterfi *et al.*, 1960), 1998, 1999 and 2000 (Gudasz *et al.*, 2000). The results of a qualitative analysis on planktonic algae from Lake Ştiucii carried out in July 2005 were also included.

Several trophic and organic pollution indices based on phytoplankton community were considered (Willen, 2000). The first one, the trophic index according to Henonen (1980) is calculated as the ratio between the number of species indicating eutrophic conditions and the number of species indicating oligotrophic conditions. The values lower than 8 show oligotrophic waters. Another trophic index is the one introduced by Nygaard (1949), which represents the number of species of Cyanoprokaryota, Chlorococcales, Centrales and Euglenophyta divided by the number of species belonging to Order Desmidiales. Values below 1 indicate oligotrophic conditions, values between 1 and 3 mesotrophic conditions and values exceeding 3 eutrophic conditions. The α and β eutrophic indices according to Oltean (1977) are calculated as follows: $I_{\alpha} = (C*\log N) / (Py+Ch+V+T+D+P+E+Cy)$; $I_{\beta}= [(C+Py) \log N] / (Ch+V+T+D+P+E+Cy)$; where N – the total number of taxa; C – Centrales; Py – Pyrophyta (Dinophyta); Ch – Chrysophyta; V - Volvocales; T - Tetrasporales; D – Desmidiales; P – Chlorococcales; E – Euglenophyta and Cy – Cyanoprokaryota (Cyanobacteria). These two indices can only be used for ecosystems where water blooms are observed. The values of I_{α} and I_{β} are inverse proportional to the water trophic level.

The organic pollution index calculated at the species level (Palmer, 1969) represents the sum of the indicator values of the species tolerant to organic load. Values not exceeding 15 indicate low organic pollution; values between 15 and 19 show moderate pollution and values greater than 20 represent high organic pollution. Reynolds (1984, 1997) proposed several functional groups of lentic algae based on representative species or genera. This system is based on a series of interrelationships between the algal morphological type, community functions and the variability of environmental factors.

Planktonic microcrustaceans (cladocerans and cyclopoid copepods) in Lake Ştiucii were sampled monthly for two years, from May 2003 to April 2005. The sampling procedures and the subsequent analyses of microcrustaceans were carried out using standardized methods (Clesceri *et al.* (ed.), 1998). The Index of Cenotic Significance (ICS) (Rogozin, 2000) was calculated in order to identify the most important cladoceran and copepod species as regards their frequency of appearance and their biomass (estimated using length-weight regression equations). The index is defined as the frequency (the percentage of samples with the species) multiplied by the square root of the mean species biomass. The index was calculated for each microcrustacean species and in the end only the species with the highest ICS values were considered.

The saprobic index according to Zelinka and Marvan (1961) was considered: $S = \Sigma(SGH)/\Sigma(GH)$, where S – the saprobic index; G – the indicator value of the species; H – the frequency of the species (ranging from 1 – species not frequent to 5 – dominant species). The interpretation of this index allows the extrapolation of saprobic classes (oligosaprobic, mesosaprobic, eusaprobic) to water quality classes: if the values are lower than 1.7, the water organic pollution is low; if the index varies between 1.8 and 2.5, the organic pollution is moderate; if the values exceed 2.6, the pollution is strong and very strong.

Biotic indices based on zooplankton species are rare. The one chosen for this paper represents the ratio between large cladocerans (C_l) and the density of all cladoceran species (C_t) (Moss *et al.,* 2003, from Török *et al.,* 2008). The values of this index indicate five water quality classes, according to the Water Framework Directive: when the values are lower than 0.2, the water quality is bad or poor; when the values vary between 0.2 and 0.5, the water quality is moderate; if they exceed 0.5, the water quality is good or high..

Results and discussion

According to the Heinonen trophic index (1980), Lake Ştiucii was oligotrophic in 1943 and 1958 (the index reached 2 and 4, respectively, both lower than 8, the reference value). The index calculated for 1998, 1999, 2000 and 2005 recorded values that exceeded 8, indicating eutrophic conditions. This particular index does not allow the differentiation of mesotrophic conditions or of the early phases of eutrophication.

The composed trophic index introduced by Nygaard (1949) reached the value 1 in Lake Ştiucii in 1943, indicating oligotrophic conditions. In 1958, its value of 1.2 showed a mesotrophic water body, while the values 10, 8, 7 and 7.1 calculated in 1998, 1999, 2000 and 2005, respectively, indicated eutrophic conditions (the values exceeded 3).

The trophic indices proposed by Oltean (1977) are based on the observation that water blooms appear in early stages of eutrophication or in true eutrophic conditions. So, these indices were calculated only for the years 1998, 1999, 2000 and 2005, when water blooms were observed in Lake Ştiucii. Oltean proved the existence of a clear succession of the algal groups causing water blooms. Thus, diatoms (Bacillariophyta) indicate the early stages of eutrophication, while Cyanoprokaryota are characteristic to high eutrophic waters (polytrophic or hypertrophic waters). This succession is not a linear process; stagnations or relapses might occur, due to natural and/or human factors.

Between 1998 and 2005, water blooms caused by diatom were recorded in spring and water blooms induced by Dinophyta in summer. Thus, α and β eutrophic indices were calculated (Table 1). Their values indicated early eutrophic conditions (meso-eutrophic) of Lake Ştiucii, because the trophic level is inverse proportional to the index value.

Table 1.

The values of the trophic indices according to Oltean (1977) in Lake Ştiucii

Trophic index (Oltean, 1977)	1998	1999	2000	2005
α eutrophic index	0.1555	0.0248	0.0332	0.0361
β eutrophic index	0.3888	0.0890	0.0948	0.0242

The results of the organic pollution (saprobity) index at the species level (Palmer, 1969) indicated a predictable evolution of Lake Ştiucii. The index values calculated for 1943 and 1958 (3 and 8, respectively) indicated low organic loads. Moderate organic pollution was indicated by the values 16, 18, 17 and 20 for the years 1998, 1999, 2000 and 2005, respectively.

As for the functional groups of planktonic algae according to Reynolds (1984, 1997), the phytoplankton community of Lake Ştiucii from 1943 and 1958 was included in the **N** and **P** types, characterized by the presence of some Desmidiaceae species belonging to *Cosmarium* and *Staurastrum* genera, and some diatom species of *Tabellaria* and *Fragilaria*. This particular type of algal community is characteristic for temperate oligo-mesotrophic lakes. On the other hand, in the years 1998, 1999, 2000 and 2005, the planktonic algal communities were dominated by Dinophyta species, with the constant presence of *Ceratium hirundinella*, typical for temperate lakes and some *Peridinium* species (*P. voltzii, P. umbonatum, P. cinctum, P. bipes* etc.). The functional group according to Reynolds (1984, 1997) was **L**. *Ceratium hirundinella* is common in stratified lakes with low nutrient content (oligotrophic) or with high nutrient content (meso-eutrophic), having the ability to move in the water column in order to use the food resources from different depths. Large *Peridinium* species (*P. voltzii* and *P. cinctum*) are characteristic to meso- and eutrophic lakes, because they can cause water blooms at any time, in association with *Ceratium hirundinella* or with some Cyanoprokariota (*Gomphosphaeria* or *Microcystis flos-aquae*).

As for zooplankton, 26 microcrustacean species (cladoceran and cyclopoid copepod) were identified in Lake Ştiucii between May 2003 and April 2005. The general structure and dynamics of microcrustacean communities in temperate water bodies include a few “common” species, with high biomass and high frequencies, which best characterize the ecological status of the lake.

According to the Index of Cenotic Significance (ICS), the dominant species in Lake Ştiucii was the cladoceran *Bosmina longirostris* (ICS = 723.86); the cladoceran *Daphnia cucullata* represented the codominant species (ICS = 585.74), while the copepods *Cyclops vicinus* and *Thermocyclops crassus* and the cladoceran *Ceriodaphnia pulchella* were the subdominant species in Lake Ştiucii during 2003 and 2005 (ICS = 377.10; 143.37 and 122.97, respectively).

These dominant, codominant and subdominant species are known to indicate relatively clean waters considering the saprobity (oligosaprobic to β mesosaprobic systems) (Table 2).

The saprobic index according to Zelinka & Marvan (1961) recorded 1.57 for Lake Ştiucii, indicating a slightly polluted environment. However, this value of the index should be regarded with caution, because not all species present in the samples had an indicator value (Sládeček, 1973), and because the index did not consider the biomass of the species identified, only their presence.

Similarly, most dominant, codominant and subdominant species from Lake Ştiucii develop in meso-eutrophic waters (Table 2).

Table 2.

List of dominant, codominant and subdominant microcrustacean species from Lake Ştiucii according to the Index of Cenotic Significance, together with the saprobic level and the trophic conditions they indicate

Species	Saprobic level / trophic conditions	Author(s)
Bosmina longirostris	oligosaprobic → β mesosaprobic	1; 2; 3
(dominant)	habitat: small eutrohic unpolluted water bodies	
Daphnia cucullata	oligosaprobic → β mesosaprobic	1; 2; 3; 4
(codominant)	habitat: mesotrophic or eutrophic waters	
Cyclops vicinus	β mesosaprobic	1; 5; 6; 7
(subdominant)	habitat: mesotrophic and eutrophic waters	
Thermocyclops crassus	oligosaprobic	5; 6
(subdominant)	habitat: mesotrophic and eutrophic water; pelagic area	
Ceriodaphnia pulchella	oligosaprobic → β mesosaprobic	1; 3
(subdominant)	habitat: eutrophic waters with submerged macrophytes	

1 - Sládeček, 1973; 2 - Negrea, 1983; 3 - Negrea, 2002; 4 - Smakulska and Gorniak, 2004;
5 -Damian-Georgescu, 1963; 6 - Pleşa and Müller, 2002; 7 - Hansen and Santer, 2003

The biotic index calculated for zooplankton was based on the ratio between large cladocerans and the total density of all cladoceran species (C_l/C_t). Large cladoceran species were considered to be those with a length greater than 700 µm (measured according to Culver *et al.*, 1985). The index recorded 0.299 for Lake Ştiucii, indicating moderate ecological status.

Conclusions

All trophic indices based on phytoplankton used in the present paper, together with the functional algal groups, suggested oligotrophic conditions in 1943-1958 and mesotrophic or early eutrophic conditions after the year 1990. The organic pollution index calculated at the species level indicated low pollution in 1943-1958 and moderated pollution after 1990.

Dominant, codominant and subdominant microcrustacean species identified in Lake Ştiucii between 2003 and 2005 also indicated meso-eutrophic conditions and low organic pollution (similarly to the saprobic index that showed a slightly polluted ecosystem). The biotic index based on planktonic cladocerans qualified once again the ecological status of Lake Ştiucii as moderate.

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ADMINISTRATION OF *SPIRULINA* TO ETHANOL-FED RATS INCREASES THE RESISTANCE OF HEPATIC MITOCHONDRIA TO CALCIUM-INDUCED PERMEABILITY TRANSITION AND LOSS OF MEMBRANE POTENTIAL

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SUMMARY. Female white Wistar rats were treated as specified below for an average period of 10 weeks. The control group (C) received a standard diet. The ethanol-fed group (E) consumed the same diet but their drinking water contained 10% (v/v) ethanol. The third group (ES) received the same treatment as E, but, in addition, the rats were supplemented with 8% Spirulina powder in their food. A fourth group received the standard diet plus the Spirulina supplement (S). At the end of the period, the rats were sacrificed and part of their liver was used for the preparation of mitochondria. Membrane potential ($\Delta\Psi$), calcium fluxes and matrix swelling were followed spectrophotometrically, in some cases by parallel recordings, using a diode array instrument, while $\Delta\Psi$ was also recorded fluorimetrically. The respiration and all the associated phenomena monitored by us were triggered by the addition of succinate in the presence of rotenone. From the comparison of the spectrophotometric recordings, we observed a good correlation between the concentration of calcium added to the mitochondrial suspension, the moments of $\Delta\Psi$ collapse, swelling and calcium release (massive efflux). The sensitivity of mitochondria to calcium was quantified in terms of 10-µM pulses added until $\Delta\Psi$ collapse and/or calcium efflux and served as a reliable index of comparison between groups. The permeability transition (PT) induced by swelling was confirmed by the inhibitory effect of cyclosporin A. Important differences were noticed between the mitochondria of the four groups regarding the kinetics of the absorbancy changes associated with the generation/collapse of $\Delta\Psi$, Ca^{2+} movements and swelling. In general, mitochondria from ethanol-treated rats (E) were more sensitive to calcium, while those of the S group were the least sensitive. The double- treated group (ES) had practically the same performance as the C group. We conclude that Spirulina utilised as a food additive significantly enhances the resistance of mitochondria to calcium-induced membrane potential collapse and permeability transition.

Keywords: ethanol-fed rats, Spirulina, mitochondrial membrane potential, calcium efflux, permeability transition.

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Introduction

Although the mechanism by which ethyl alcohol (ethanol) exerts its deleterious effects on liver, inducing the so-called alcoholic liver disease (ALD) is not entirely known, chronic ethanol exposure has been constantly associated with mitochondrial oxidative stress (OS) and partial decrease in ATP synthesis (Kukielka *et al.*, 1994; Halestrap *et al.*, 1997; Kurose *et al.*, 1997; Bailey *et al.*, 1999; Adachi and Ishii, 2002; Cunningham and Van Horn, 2003; Wu and Cederbaum, 2003; Albano, 2006; Das and Vasudevan, 2007; Manzo-Avalos and Saavedra-Molina, 2010). On the other hand, the same type of agents are implicated in membrane permeabilization and release of the so-called apoptogenic factors (*i.e.,* biological molecules able to induce apoptosis), which usually reside in the mitochondrial intermembrane space (Kroemer *et al.*, 1995; 1998; Marchetti *et al.*, 1996; Chernyak, 1997; Scarlet and Murphy, 1997; Hirsh *et al.*, 1998; Bernardi *et al.* 1999; 2001; Crompton, 1999; Pastorino *et al.*, 1999; Newmayer and Fergusson-Miller, 2003; Kim *et al.*, 2003; Le Bras *et al.*, 2005; Yan *et al.*, 2007; Jones *et al.*, 2010). Many researchers agree that the central event in this release is a drastic change in mitochondrial membrane permeability associated with the formation of a so-called permeability transition pore (PTP), a very likely irreversible phenomenon (Kroemer *et al.*, 1995; 1998; 2007; Bernardi *et al.*, 1999; Crompton, 1999; Petronilli *et al.*, 2001). Nevertheless, the exact sequence of events leading to PTP formation continues to be disputed. One point of view holds that the opening of the pore is accompanied by membrane potential collapse, uptake of electrolytes and water, matrix swelling and rupture of the mitochondrial outer membrane. As a consequence, several factors present in the intermembrane space, among which cytochrome c (cyt c), are liberated into the cytosol. There is, however, an alternative point of view, which considers that cyt c and probably other proapoptotic factors can be released without a permeability transition and outer membrane breaking, due to certain pores created into the outer membrane by several proapoptotic agents, such as Bax and Bid (Eskes *et al.*, 1998; Martinou *et al.*, 1999; von Ahsen *et al.*, 2000; Gogvadze *et al.*, 2001). Moreover, an extensive permeabilization of the mitochondrial membranes, such as that induced by PTP opening, would completely compromise the process of oxidative phosphorylation and this should affect the apoptotic process itself (which is a controlled process, needing ATP), The result would be death by oncosis (*i.e.*, having to do with swelling), better known as necrosis, although oncosis is a more appropriate term. Indeed, in many instances, ALD is better characterized by extensive swelling of the hepatic tissue and cell death by necrotic oncosis (necrosis) rather than by apoptosis (see, for example, Kim *et al.*, 2003), although the contribution of the two processes is difficult to separate.

From a practical point of view, it is important to know which factors can promote ALD and which can protect the liver. From the above presentation, it is clear that everything that contributes to mitochondrial membrane permeabilization (leading to apoptosis and/or necrosis) would aggravate ALD, whereas factors that prevent or at least decrease the extent of this phenomenon would be protective. As mentioned before, oxidative stress (*i.e.*, an imbalance in the redox state of the antioxidant system of the cell, following an excessive production of oxidant chemical species, such as reactive oxygen and nitrogen species) has been associated with ALD. In the eukaryotic cell, the major producer of reactive oxygen species (ROS) is the mitochondrial respiratory chain (see reviews of Valentine *et al.*, 1998; Barja, 1999; Muller, 2000; Turrens, 2003; Orrenius *et al.*, 2007). However, under normal conditions, ROS production is limited to what is needed in the cell signalling pathways. Any factor or condition leading to an increased production of ROS will contribute to the generation of an oxidative stress that promotes lipid peroxidation, formation of protein adducts and DNA breaks as well as serious dysregulations in cell signalling pathways, which, in turn, will exacerbate ROS production and so on (Cross *et al.*, 1987; Kourie, 1998; Bandyopadhyay *et al.*, 1999; Hensley *et al.*, 2000; Robertson and Orrenius, 2000; Hancock *et al.*, 2001; Le Bras *et al.*, 2005; Zorov *et al.*, 2006; Bayir and Kagan; 2008). It is expected for the calcium ions, which are also very much involved in both mitochondrial metabolism and a series of cell signalling pathways (Hoek *et al.*, 1995; Robb-Gaspers et al., 1998; Duchen, 2000; Rizzuto *et al.*, 2000; Carafoli, 2002; Ermack and Davies, 2002; Brookes *et al.*, 2004; Giacomello *et al.*, 2007; Adam-Vizi and Starkov, 2010) to be very important in this positive feed-back loop This is the reason why Ca^{2+} fluxes at the level of the inner mitochondrial membrane are finely regulated (Bernardi and Pietrobon, 1982; Bernardi and Azzone, 1983; Saris, 1997; Duchen, 2000; Carafoli, 2002; Orrenius *et al.*, 2003). Under the conditions of OS, this regulation is altered due to increased membrane permeability induced by ROS. In fact, reactive oxygen species damage all mitochondrial components (membrane lipids and proteins, intermembrane and matrix proteins, mitochondrial DNA). Thus, in a similar way to what we have described above for the ROS-stimulated ROS generation, the loss of calcium control stimulates ROS production and *vice-versa* (Richter, 1997; Tan *et al.*, 1998; Grijalba *et al.*, 1999; Rizzuto *et al.*, 2000; Orrenius *et al.*, 2003; Batandier *et al.*, 2004; Yan *et al.,* 2007; Giacomello *et al.*, 2007; Kroemer *et al.*, 2007; Peng and Jou, 2010). Magnesium, on the other hand, has been shown to have a protective effect on mitochondrial behaviour with respect to calcium fluxes, increasing membrane resistance to permeability transition (Novgorodov *et al.*, 1994; Kowaltowski *et al.*, 1998; Günther, 2006; Racay, 2008; Romani, 2008).

Cyanobacteria of the genus *Arthrospira* (formerly known as *Spirulina*) are recognised for their use as a supplement in human and animal nutrition. The chemical composition of the *Spirulina* powder (a commercial product to which we will be referring simply as Spirulina) indicates the presence of phenolic acids, tocopherols and β-carotene, which are known to exhibit antioxidant properties. Indeed, there are several studies which confirm the antioxidant properties of Spirulina *in vivo* and *in vitro* (Bhat and Madyastha, 2000; 2001; Wu *et al.*, 2005; Riss *et al.*, 2007; Dartsch, 2008). Spirulina also possesses potent antiviral, anticancer, immunostimulating and metalloprotective effects and its safety for human consumption has been established through numerous toxicological studies (Belay *et al.*, 1996; Belay, 2002; Blé-Castillo *et al.*, 2002; Khan *et al.*, 2005; Amin *et al.*, 2006; Mohan *et al.*, 2006; Ismail *et al.*, 2009; McCarty *et al.*, 2009).

The purpose of the present study was to establish whether Spirulina has a protective effect on liver mitochondria of both normal and ethanol-fed rats with regard to the permeability transition and associated phenomena (matrix swelling, $\Delta\Psi$ collapse and calcium efflux). This represents a preliminary report from a series of results that are part of a larger study which is trying to evaluate the use of Spirulina as a protective nutritional factor in alcoholism and to elucidate the mechanisms by which this food additive exerts its hepatoprotective effects.

Materials and methods

Animals, treatment protocols and preparation of mitochondria. The animals used in this study were female Wistar rats, weighing at the end of the treatment period 170±20 g. The rats were divided randomly in four groups: the control group (**C**) was fed a standard diet (a premix containing all the ingredients of the Larsen diet), with free access to water; the rats in the ethanol-fed group (E) had a similar diet, but their drinking water contained 10% (v/v) ethanol; the third group (ES) received the same treatment as the E group, but, in addition, the rats were supplemented with 8% Spirulina powder; a fourth group (S) received the standard diet plus the Spirulina supplement. At the end of the treatment period (10±1 weeks), the animals were fasted for 24 hrs and sacrificed through exsanguination, after a slight anaesthesia, and blood and liver tissue samples were taken to determine specific haematological, biochemical and enzymatic parameters, as well as tissue samples for electron microscopy. The remainder of the liver was homogenized for mitochondrial preparations The present article describes only the results obtained with mitochondria. These organelles were isolated by differential centrifugations, essentially as previously described (Johnson and Lardy, 1967; Tarba, 1983b), in a medium containing 200 mM mannitol, 70 mM sucrose, 5 mM Hepes-KOH (pH 7.38) and 0.5 mM Na-EDTA. The washing and preserving medium lacked the chelating agent (EDTA).

Spectrophotometric measurements. Membrane potential ($\Delta\Psi$), calcium fluxes and matrix swelling were monitored spectrophotometrically, as also described (Tarba and Suărăşan, 2004), either using a diode array instrument (Specord S 100, Analytik Jena, Germany), which allows parallel recordings of several parameters, or using a Jasco V-530 spectrophotometer and performing individual recordings of each parameter under identical conditions. In addition, in the majority of the cases, the membrane potential was also followed spectrofluorimetrically (with Jasco FP-750). In all cases, 1 mg of mitochondrial protein/ml was incubated directly in the spectrophotometer or spectrofluorimeter cuvette(s), at room temperature (around 24^{O}C), in a medium usually containing 100 mM mannitol, 35 mM sucrose, 70 mM KCl, 2,5 mM KP_i, 1,5 mM $MgCl_2$ and 5 mM Hepes buffer (pH 7.38). 10 µM rotenone was usually added to the mitochondrial suspension, as well as the appropriate probes for either $\Delta\Psi$, *i.e.*, 2.5 µM of the cationic cyanine dye 3,3'-diethylthiadicarbocynine (usually known by the acronym diS-C_2-(5)) or for Ca^{2+} (30 µM arsenazo III). The respiration and all the associated phenomena monitored by us (ΔΨ, Ca^{2+} fluxes and swelling) were triggered by the addition of succinate (2.5 mM). Different amounts of $CaCl_2$ or other modulating factors were either added gradually, in several pulses, or in one pulse, up to the desired final concentration. The exact conditions of representative recordings are described in the corresponding figure legends.

Statistical analysis. The sensitivity of mitochondria to calcium was quantified in terms of 10-μM pulses added until ΔΨ collapse and/or calcium efflux and served as a reliable index of comparison between groups. The comparison was performed using a two-factor ANOVA procedure followed by the Student-Newman-Keuls test, in the post-hoc analysis, for establishing the statistical significance of the differences between group means.

Results and discussion

Mitochondria of the control (C) rats. Representative examples for the behaviour of the control mitochondria (C), regarding the kinetics of membrane potential, calcium fluxes and swelling, under stress conditions induced by calcium, are presented in Figs.1-5. Figs 1 and 2 are examples of simultaneous recordings with the diode array spectrophotometer. In Fig 1, ΔΨ is recorded in parallel with the matrix swelling, whereas in Fig 2 calcium fluxes and swelling are recorded in parallel. In principle, one could record all 3 parameters simultaneously, but, due to the fact that the spectral changes of the two probes display a rather extensive degree of overlapping, we preferred the simpler and clearer variant, using the absorbance changes associated with swelling to connect the other two parameters (ΔΨ and calcium fluxes). The upper trace in Fig 1 represents a difference recording (670 – 700 nm) and illustrates the kinetics of ΔΨ, while that in Fig 2 is a difference recording for calcium fluxes (650 – 700 nm). The lower curves in both figures represent absorbance decreases at 700 nm associated with the matrix swelling, 700 nm being a quasi-isobestic point for spectral changes of both dyes. The “spikes” on these lower traces reflect the electronic noise associated with succinate and Ca^{2+} additions to the cuvettes and represent a good mark for the exact moments of the addition.

Usually, the swelling can be monitored at 540 nm, where the spectral changes are more extensive, and this is illustrated in Fig 3. One can see, however, that the lower curves in Figs 1 and 2 have the same sigmoidal shape as in Fig 3, a feature reflecting the individual opening of the permeability transition pores of the mitochondrial population over a certain period of time, depending on incubating conditions, as previously discussed (Tarba and Suărăşan, 2004; Tarba, 2009). Another interesting feature is present in Fig 2, where one can notice the “saw tooth” behaviour in the upper trace associated with the gradual addition of 10-μM calcium pulses and their consecutive uptake by mitochondria. This behaviour repeats as long as swelling is minimal and ΔΨ has not significantly decreased.

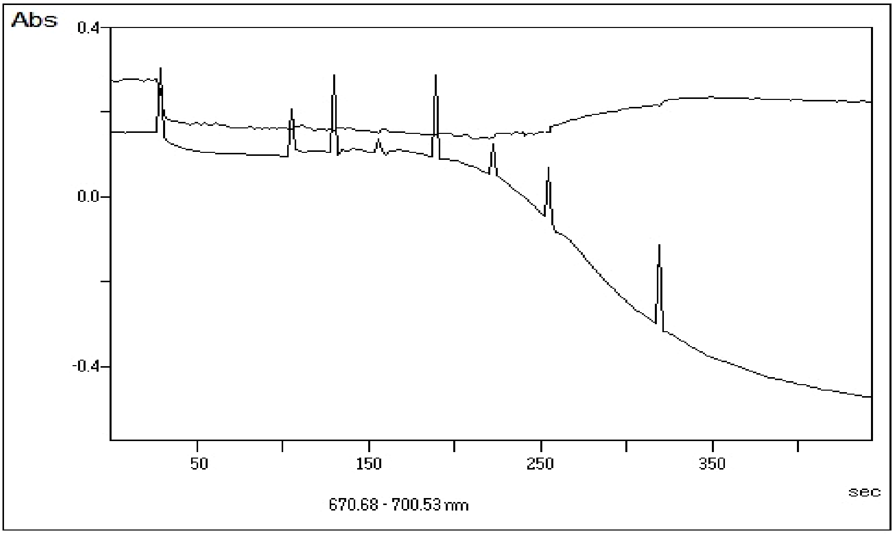

Fig. 1. ***Membrane potential (upper trace) and mitochondrial swelling (lower trace) in mitochondria of control (C) rats.*** 1 mg/ml mitochondrial protein is suspended in 100 mM mannitol, 35 mM sucrose, 70 mM KCl, 2.5 mM Pi, 1.5 mM $MgCl_2$ and 5 mM Hepes, pH 7.38, in the presence of 10 μM rotenone and 2.5 μM diS-C_2-(5). The first artefact (spike) is associated with succinate addition (2.5 mM) and the rest with 10 μM-Ca^{2+} pulses.

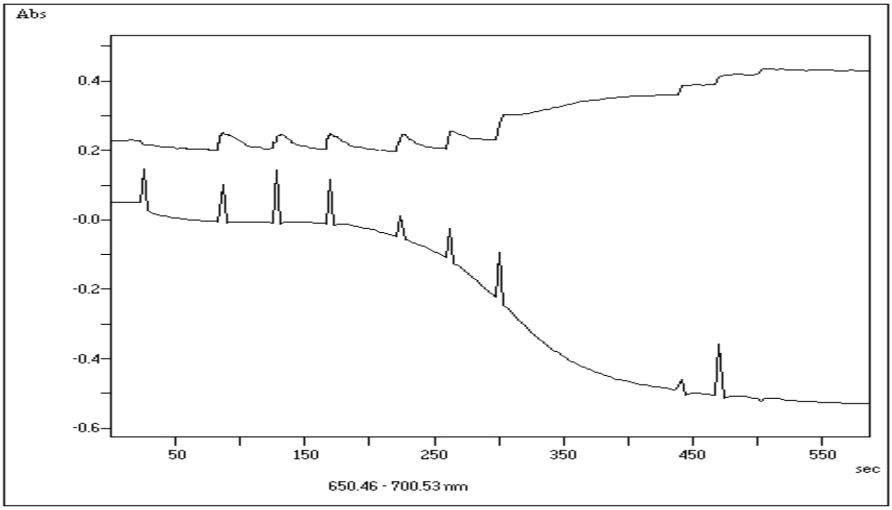

Fig. 2. ***Calcium (Ca^{2+}) fluxes (upper trace) and matrix swelling (lower trace) in mitochondria of C rats.*** The conditions and the explanations are the same as in Fig.1, except that 30 μM arsenazo III is used instead of diS-C_2-(5). The first six Ca^{2+} pulses are 10 μM each, in order to facilitate comparison with the situation in Fig.1.

This is in agreement with the way in which calcium is taken up by mitochondria, by mechanisms driven by ΔΨ, particularly by a calcium uniporter (Günther and Günther, 1994; Bernardi, 1999; Kirichok *et al.*, 2004).

The number of "teeth" recorded in C mitochondria under our standard conditions varies from 4 to 8, with a mean slightly above 5 (as we will show in a latter subsection), after which the absorbance increases gradually in a hyperbolic fashion, indicating the release of Ca^{2+} into the medium (massive calcium efflux) associated with the permeability transition. Indeed, while the swelling approaches the inflexion point of the sigmoidal curve, ΔΨ collapses (see Fig.1) and the massive efflux of calcium can be observed (Fig.2). It is clear from such recordings that the 3 phenomena are tightly interconnected.

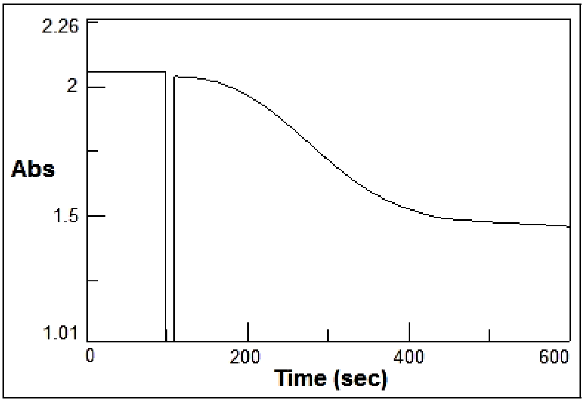

Fig. 3. ***Typical sigmoidal curve of absorbancy changes associated with mitochondrial matrix swelling upon succinate addition.*** Suspending conditions are similar to those in Figs.1 and 2, but mitochondria are present only in the sample cuvette and no dye is added. To increase the speed of swelling, 50 µM $CaCl_2$ are also present in the suspending medium. The recording was performed at 540 nm with a double-beam spectrophotometer.

Until the collapse of ΔΨ, much more discrete changes can be observed on membrane potential trace in Fig.1. This is so because ΔΨ changes little upon addition of relatively small quantities of Ca^{2+}, while the response mechanism of the potential sensitive probe itself is not linear but sigmoidal (Tarba, 1983a; 1983b). Only when the maximum loading capacity of the mitochondria has been reached, the structural and functional properties of mitochondria change abruptly. Most likely, this is the moment of PTP opening or its jump from a low conductance to a high conductance state. The fact that the swelling is a consequence of PTP opening or of the changing of its state and not of some unspecific increase in membrane permeability can be tested by the use of cyclosporin A, a specific inhibitor of pore opening (Broekemeier *et al.*, 1989). Indeed, from Fig.4, it can be seen that the addition of 1 μM cyclosporin A allows mitochondria to accumulate much more calcium before extensive swelling and calcium efflux ensue. More exactly, in the presence of cyclosporin A, mitochondria are able to accumulate over 200 nmoles Ca^{2+}/mg protein (Fig.4), as compared to 50 nmoles in the absence of cyclosporin A (Fig.2).

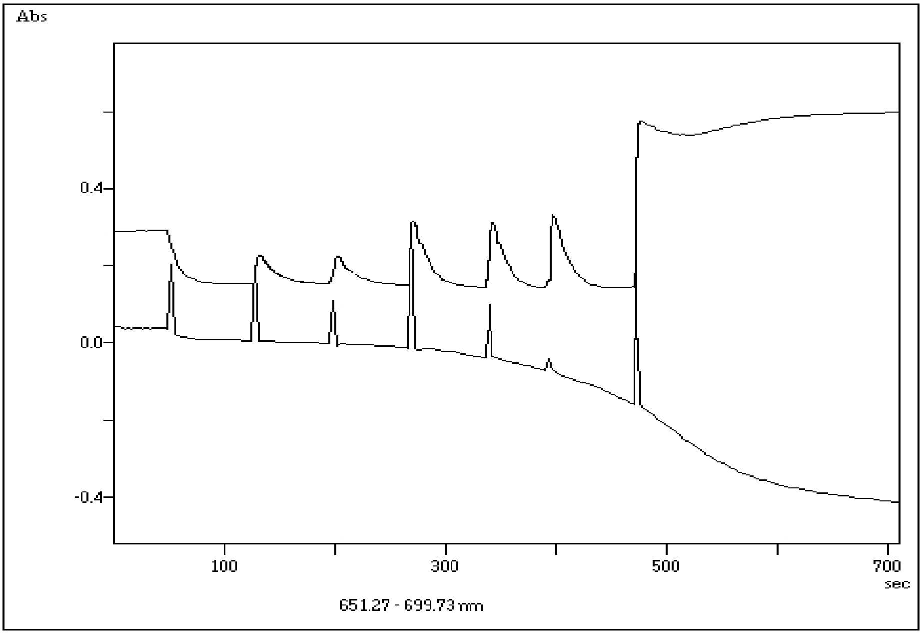

Fig. 4. ***Calcium fluxes (upper trace) and mitochondrial swelling (lower trace) in the presence of cyclosporin A.*** The conditions are similar to those in Fig.2, except that 1 μM cyclosporin A and 25 μM $CaCl_2$ are added to the mitochondrial suspension before succinate addition (first spike). The rest of the spikes.mark the addition of calcium in the following concentrations: two pulses of 10 μM, three pulses of 25 μM and one pulse of 100 μM.

Once the tight connection (correlation) of the 3 phenomena has been demonstrated, for the rest of the figures in this article we will prefer to present single recordings of calcium fluxes and ΔΨ, obtained with the aid of an usual (double-beam) spectrophotometer and a spectrofluorimeter, respectively. Fig.5 presents a recording of fluorescence changes (λ_{ex} = 622 nm; λ_{em} = 670 nm) associated with the generation and collapse of the membrane potential following succinate addition and repeated calcium pulses, respectively. Fluorescence recording has the advantage of not being sensitive to spectral changes associated with swelling, while all the additions are made in only one cuvette. By comparing Figs 1, 2 and 3, one can see that the number of "calcium teeth" correspond practically with the number of calcium additions after which ΔΨ collapses. This is to our advantage, because we can quantify the capacity of mitochondria to resist to calcium-induced stress just by inspecting the membrane potential recordings with the fluorescence method (the simplest and most reliable).

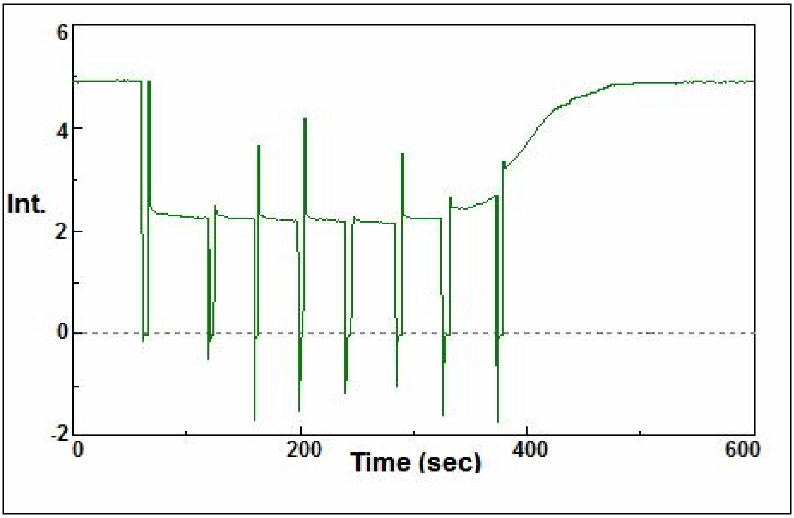

Fig. 5. ***Spectrofluorimetric recording of membrane potential kinetics upon addition of succinate and several 10-µM calcium pulses.*** Suspending conditions are similar to those in Fig.1; λ_{ex} = 622 nm and λ_{em} = 670 nm.

Mitochondria of the ethanol-fed (E) rats. Before presenting the recordings of this group, we have to make the observation that of the 8 treated animals only 7 survived and only 5 behaved in a consistent manner, characteristic for alcohol-fed rats, while 2 had a totally different behaviour, their performance (i.e., resistance to calcium-induced stress) being even above the control mean (see the last subsection of our results). A similar situation has been observed by us before, in functional and ultrastructural studies on mitochondria of rats fed ethanol by a different procedure, and its biological significance was discussed (Tarba and Suărăşan, 2004; Tarba and Florea, 2006). The general observation that not all the rats respond identically to ethanol has in fact a rather long history (see Mardones and Segovia-Riquelme, 1983). The quantitative measurements that we are going to present later in this article will prove that the two (sub)groups differ significantly from a statistic point of view. Therefore, in the rest of our presentation we will refer to the E group as composed of the 5 individuals with a typical response to ethanol intoxication.

Figs. 6 and 7 illustrate the kinetics of $\Delta\Psi$ and of calcium fluxes, respectively, of a representative preparation in the E group of 5 rats. It can be seen that mitochondria of this group are more sensitive to calcium-induced stress than the control ones, as demonstrated by the smaller number of 10-µM calcium pulses added until the collapse of $\Delta\Psi$ (Fig.6) or the release of Ca^{2+} (Fig.7). These results point to the fact that the mitochondrial membranes of the alcoholic rats and their transport systems are more fragile, ready to give up to slight or moderate metabolic stresses and release the components of the intermembrane space and probably even of the matrix, despite the apparent well-being of the animals. The fact that one of the animals in the initial E group died is likely to be related to a higher individual sensitivity of that rat to ethanol. It is also worth mentioning that females, in both rodents and humans, are more sensitive to ethanol than males (Arthur *et al.*, 1984; Vetter-O'Hagen *et al.*, 2009).

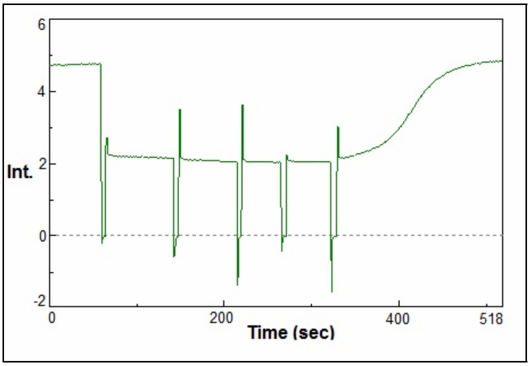

Fig. 6. ***Spectrofluorimetric recording of membrane potential generated by succinate-induced respiration in mitochondria of ethanol-fed (E) rats.*** Suspending and recording conditions are identical to those in Fig.5.

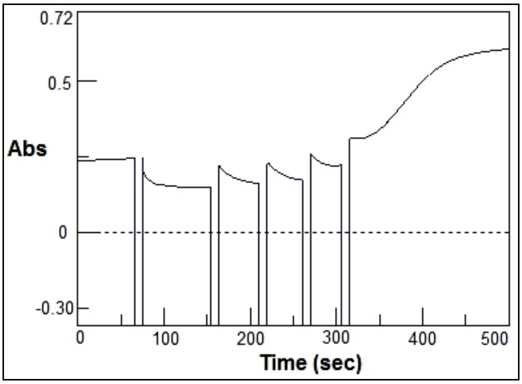

Fig. 7. ***Calcium fluxes in mitochondria of ethanol-fed (E) rats.*** 1mg/ml mitochondrial protein was present in each cuvette, arsenazo III only in the sample cuvette and the rest of the additions were made simultaneously in both cuvettes: first artefact marks the addition of succinate, while the rest represent additions of 10-µM calcium pulses.

Mitochondria of the double-treated (ES) rats. Representative behaviour of the mitochondria isolated from rats treated with both ethanol and Spirulina are shown in Fig.8 (ΔΨ) and Fig.9 (calcium fluxes). Their behaviour is practically indistinguishable from that of the control mitochondria, a statement which will be supported by the statistical analysis performed in the last subsection devoted to our results. However, we have to stress the fact that the inter-individual variation of the results was the highest in this group. This is expected, since we are dealing with the effect of a combination of two factors superimposed on the normal inter-individual variability. Nevertheless, it is apparent from the recordings that Spirulina supplement has a protective effect on the liver of ethanol-fed rats.

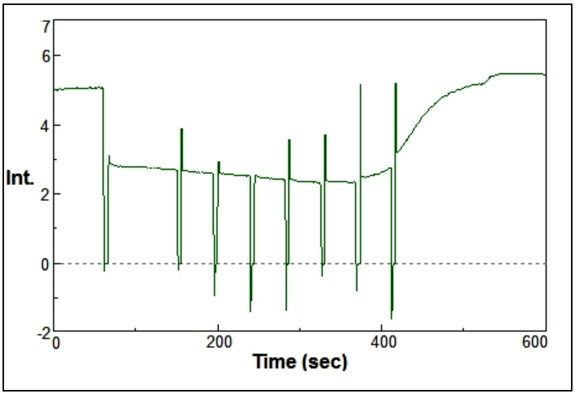

Fig. 8. ***Spectrofluorimetric recording of membrane potential generated by succinate-induced respiration in mitochondria of ethanol-fed Spirulina-treated (ES) rats.*** Suspending and recording conditions are identical to those in Fig.5.

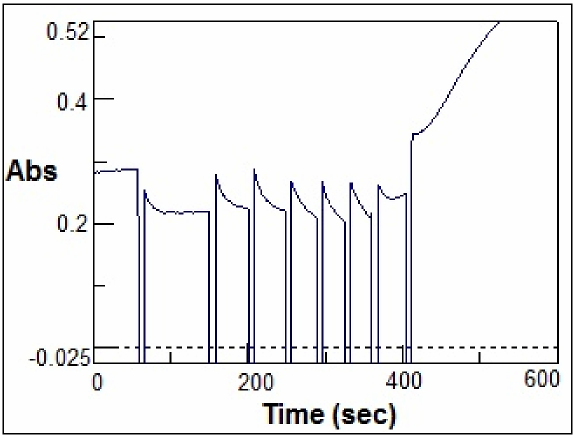

Fig. 9. ***Calcium fluxes in mitochondria of ethanol-fed Spirulina-treated (ES) rats.*** The working conditions are identical to those in Fig.7.

*Mitochondria of the Spirulina-treated (S) rats. T*wo representative illustrations for the behaviour of mitochondria isolated from this group are presented in Fig.10 (for ΔΨ) and Fig.11 (for calcium fluxes). One can see that mitochondria of this group accumulate around 6-7 calcium pulses before the induction of the permeability transition (PT), amounting to a total of 60-70 μM, or 60-70 nmoles/mg protein, the individual values varying between 45 and 90 nmoles/mg, with a mean of 66.1 nmoles/mg mitochondrial protein. Under the conditions used by us, in which inorganic phosphate (Pi) has a higher concentration than magnesium, this performance is quite remarkable, since it is known that Pi promotes calcium-induced PT, while magnesium has an opposite effect (Bernardi and Pietrobon, 1982; Novgorodov *et al.*, 1994; Kowaltowski *et al.*, 1998; Gunther, 2006; Racay, 2008). This means that Spirulina added as a nutritional supplement increases the resistance of liver mitochondria to calcium-induced stress and very likely to oxidative stress, since the two agents are inter-related (inter-conditioned), as described in the "Introduction". In fact, a preliminary analysis of the biochemical results in our larger study (unpublished results) points into this direction.

Quantitative comparison regarding the sensitivity of mitochondria to calcium. As mentioned before, if we take into consideration the number of calcium pulses (10 μM each or 10 nmoles/mg protein) necessary to induce calcium release or ΔΨ collapse, the sensitivity of mitochondria can readily be quantified using relatively small integers or fractions equal to 0.5 and computing means that can be compared by the use of the Student *t* test or other more sophisticated tests. The first comparison of this kind that is presented in Table 1 proves that the ethanol-fed group (E) did not respond in a homogeneous manner to ethanol treatment and that there are statistically significant differences between two subgroups, E_1 and E_2.

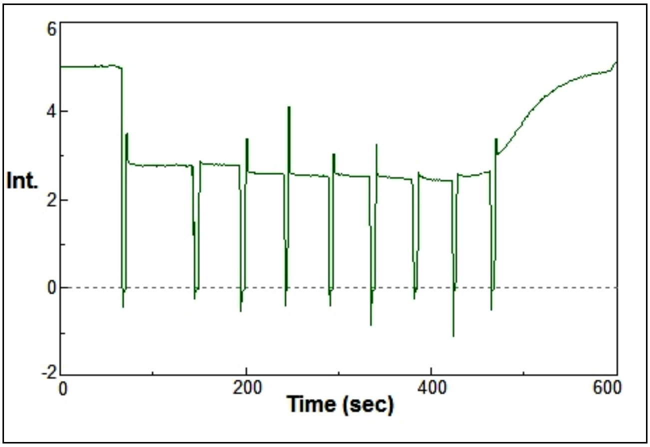

Fig. 10. ***Spectrofluorimetric recording of membrane potential generated by succinate-induced respiration in mitochondria of Spirulina-treated (S) rats.*** Suspending and recording conditions are identical to those in Fig.5. Note the increased number of 10--μM calcium pulses before membrane potential collapse.

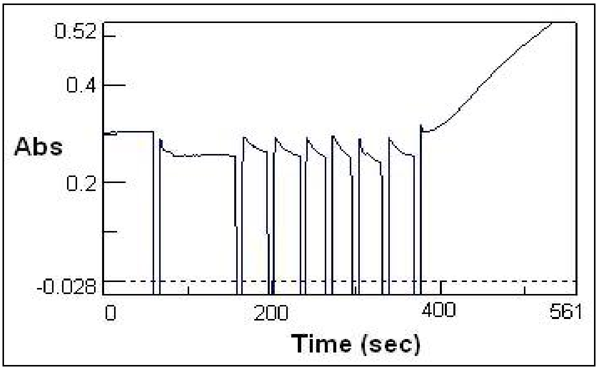

Fig. 11. ***Calcium fluxes in mitochondria of Spirulina-treated (S) rats.*** The working conditions are similar to those in Fig.7.

Table 1

Different response to ethanol feeding of two rat subgroups (E_1 and E_2)

Subgroup	Number of individuals	Mean ± SE_M	t	p
E_1	5	3.40 ± 0.19		
E_2	2	6.50 ± 0.50	7.56	< 0.0001

From this table, we can see that the differences between the two means, according to the *t* test, are highly significant. Under these circumstances, it is recommended to consider the two subgroups as distinct and to compare them to the control. By doing this, we established that there is no statistical difference between E_2 and the control (or any other group). Consequently, we assimilated E_1 with E (new group of only 5 rats). Since the total number of comparisons that can be made in the case of 4 groups is 6 [*i.e.*, (4 – 1)(3 – 1)(2 – 1)], an ANOVA procedure and a post-hoc multiple comparison test is recommended, in order to avoid the unjustified rejection of the null hypothesis. Due to the fact that we are testing the effect of two factors, acting in one case together, a two-way or two-factor ANOVA was used. For this purpose, we set up a preliminary table, in which every group appears with the corresponding number of rats and mean. In addition, the number of rats in each row and each column, plus the grand total, are also included. The resulting situation is presented in Table 2.

Table 2

Preliminary table for the two-factor ANOVA with 4 groups (C, E, S and ES)

Group	Number of individuals	Mean	Group	Number of individuals	Mean	Row totals
C	10	5.40	S	9	6.61	19
E	5	3.40	ES	8	5.62	13
Column totals	15			17		
Grand total	32					

After computing all the possible sources of variation, in terms of the sums of squares (SS) of the deviations from means, different types of variances (mean square errors – MSE) have been computed by dividing each SS by the corresponding degree of freedom (DF) and the appropriate variance ratios (F) were calculated, by dividing each variance with the error variance. Comparing now the calculated F values with the theoretical values of the F distribution, we established that there is a very significant difference among the groups ($p < 0.01$) and significant differences between the rows and between the columns ($p < 0.05$), whereas there was no significant interaction between the two factors (ethanol and Spirulina). The results of this two-factor ANOVA are presented in Table 3. The results in the table tell us that marked differences between the groups exist, but do not tell which pairs of means actually differ. For this purpose, we used the Student-Newman-Keuls multiple comparison test and obtained the results presented in Table 4. These final results tell us that there is a very significant difference ($p < 0.01$) between the Spirulina-treated and the ethanol-fed group and significant differences ($p < 0.05$) between either control or double-treated (ES) group and the ethanol-fed group. No significant differences were detected among the other 3 groups (C, S and ES), although the S group consistently showed higher values of the parameter tested. The lack of statistical significance is most likely due to the large inter-individual variations, especially in the ES group and to some extent in the C group.

Table 3

Details of the basic results of the two-factor analysis of variance (ANOVA)

Type of variation	Sums of squares (SS)	Degrees of freedom (DF)	Variance (MSE = SS/DF)	Variance ratio (F)	p
Among groups	33.64	3	11.213	5.269	<0.01
Between rows	11.19	1	11.19	5.258	<0.05
Between columns	15.93	1	15.93	7.486	<0.05
Interaction	6.52	1	6.52	3.064	N.S.
Error	59.60	28	2.128	-	-
TOTAL	93.24	31	-	-	-

Table 4

Post-hoc analysis of the results by Student-Newman-Keuls multiple comparison test

	Group	Mean	Difference vs. E	Theoretical Q	Calculated R	p
1	S	6.61	3.21	$Q_{(0.01,4,28)}=4.83$	2.439	<0.01
2	ES	5.62	2.22	$Q_{(0.05,3,28)}=3.50$	2.114	<0.05
3	C	5.40	2.00	$Q_{(0.05,2,28)}=2.90$	1.269	<0.05
4	E	3.40	-	-	-	-

This quantitative analysis confirms the conclusions drawn from comparing the spectrophotometric and spectrofluorimetric recordings, *i.e.*, the alcoholic rats are characterised by a higher sensitivity to metabolic stress, specifically to that exerted by an increased calcium concentration, which is in agreement with our previous observations on isolated mitochondria from ethanol-fed rats (Tarba and Suărăşan, 2004) and on hepatic tissue (Tarba and Florea, 2006). Our own studies of the effects of alcohol and of several other chemical agents on the mitochondrial functions and structure (Tarba and Orbai, 1986; Tarba and Crăciun, 1990; Petrescu and Tarba, 1997) as well as other similar studies reported in the literature (Cunningham *et al.*, 1990; Fernandez-Checa *et al.*, 1991; Hoek, 1994; Pastorino *et al.,* 1999; Adachi and Ishii, 2002; Albano, 2006; Yan *et al.*, 2007; Jones *et al.*, 2010; King *et al.*, 2010) indicate an increased membrane permeability with the consecutive impairment of oxidative phosphorylation and ATP production. However, many of the studies reported in the literature, with direct relevance to the present work, refer to the perfused liver, hepatic tissue or cell cultures, while fewer studies similar with ours have been performed on isolated mitochondria. Although the experimental approach was usually not identical, the general conclusions were similar, *i.e.*, even moderate chronic alcohol consumption affects the production of ATP and induces serious functional and structural alterations in the mitochondria of vital organs and tissues, such as liver, pancreas, heart, brain, kidneys and even muscles (Kedderis, 1996; Cunningham and van Horn, 2003; Wu and Cederbaum, 2003; Albano, 2006; Das and Vasudevan, 2007; Adam-Vizi and Starkov, 2010; Jones *et al.*, 2010; Manzo-Avalos and Saavedra-Molina, 2010)

Even much fewer studies of this type have been performed with Spirulina. Most of the studies are clinical, while the experimental ones are mostly histological and biochemical, confirming the antioxidative properties of Spirulina or of some of its components, such as phycocyanin and phycocyanobilin (Blé-Castillo *et al.*, 2002; Remirez *et al.*, 2002; Patel *et al.*, 2006; Riss *et al.*, 2007; Dartsch, 2008). In general, Spirulina is presented in the literature as a wonder nutritional supplement having hepatoprotective, cardioprotective, renoprotective, antiproliferative, antiviral, anti-inflammatory and immunostimulant properties (Wu *et al.*, 2005; Mohan *et al.*, 2006; Ismail *et al.*, 2009; Deng and Chow, 2010). Most of the studies insist on the antioxidative properties of this supplement, and, since alcohol-induced oxidative stress has been amply documented (see the “Introduction”) we are tempted to explain Spirulina's effects through its antioxidative properties. However, the situation may not be so simple. It is also known that Spirulina has a rather high concentration of magnesium and, as already discussed before, there is plenty of evidence that Mg^{2+} protects mitochondria from PT and $\Delta\Psi$ collapse. Moreover, magnesium is involved in over 300 biochemical reactions in the cell, including the production of ATP by the mitochondrial ATP synthase, so additional Mg^{2+} may also contribute to the limitation of the oxidative stress and of other types of biological stress.

Conclusions

Our present results confirm the beneficial effects of Spirulina administration to both normal and ethanol-fed rats. We demonstrated that Spirulina enhances the resistance of liver mitochondria to calcium induced stress, postponing $\Delta\Psi$ collapse and permeability transition, factors involved in cell death by both apoptosis and necrosis. This is very likely an important mechanism (if not the most important one) through which Spirulina exerts its hepatoprotective effects reported in the literature.

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===SHORT COMMUNICATION===

EXPERIMENTAL RESEARCH OF BONE DISTRACTION *IN VITRO*

OVIDIU MUREŞAN[1,✉]

SUMMARY. The purpose of the study is to assess the optimal distraction rate using a lab rat model mandible in an in vitro environment, to observe neoosteogenesis in the distracted callus, without the influence of the vascular system of the bone. Results show that a distraction rate between 0.5 and 1mm per day are optimal. Different types of bone formation occur and prevail, depending on the daily distraction rate.

Keywords: bone distraction, *in vitro* osteogenesis, distraction rate.

Introduction and aims

Bone distraction has proved itself as a valuable method for generating new bone needed for reconstruction after various surgical ablations, trauma, or other lesions that involve lack of bone or deformities. First used in orthopedics, it was soon adopted for oral and maxillofacial purposes. Complex problems arise from the fact that cranial bones have a more complex structure, shape and embryology compared to the long bones of the limbs. Thus, understanding the nature of osteogenesis, bone repair, bone reshaping are essential data for further improving the use of this technique: improving the rate of success and minimizing the postoperative complications.

The purpose of the present study is to assess the optimal distraction rate in an in vitro environment, without the influence of the vascular system on neoosteogenesis.

Material and methods

A number of 8 adult lab rats (Wistar and Nordica) were included in this study. They were subjected to the same surgical protocol: under general anesthesia they underwent osteotomy of the mandible, between the second and third molar, and bone fragments were immobilized in the normal position for seven days needed for periosteal healing and fibrous callus formation.

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Then, all the mandibular fragments were harvested from the animal subjects and kept in a nurturing solution, containing bovine fetal serum, antibiotics, corticoids, vitamin C and beta-glycerol phosphate (Fig.1).

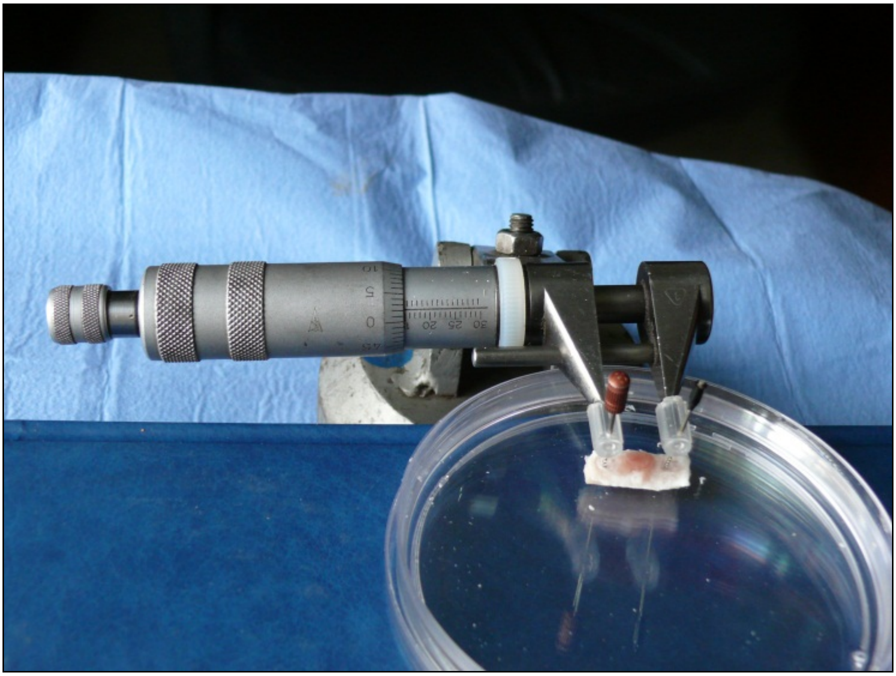

Fig.1 Bone fragment in experimental distractor with micrometric advancement, immersed in nurturing solution.

Randomly divided into 2 equal groups, the rat mandible fragments were subjected to gradual distraction for six days, at rates of 0.5mm and respective 1mm daily.

At the end of the distraction period, all fragments were prepared for microscopic evaluation and colored with hematoxylin and eosin.

Results

Microscopic examination of the distracted rat mandible fragments revealed in the 0.5mm per day group the rearranging and reorientation of cells in column-like shapes, suggesting the common origin from one single mesenchymal cell (Klein and Hovaldt, 1995; McCarthy et al., 1992). Observing sequential sections revealed the transition from condroid, to cartilaginous osteoid and then to woven bone, also revealing hypertrophic chondrocytesabout to differentiate into osteoblasts and osteocytes.

Microscopic findings in the 1mm per day group showed spindle like cells resembling to fibroblasts, secreting parallel collagen fibers that served as a matrix for bone formation. Thus multiple trabeculae extend between the two bone fragments starting from the bone surfaces and progressing towards the center of the gap aligned along the direction of the traction force. These can be regarded as signs of direct bone formation along the collagen fibers formed between the bone fragments during callus distraction.

Results were significantly similar between bone and callus samples in the same group.

Discussions

Bone distraction is achieved by applying forces to bone fragments adjacent to a callus (Ilizarov, 1971; 1988). By gradual linear tension in the callus is subjected to constant mechanical, functional linear stress, generating an environment that is very favorable to enhancing and stimulating osteogenesis, as well at a cellular, as at a subcellular level (Ilizarov, 1989a, b). The conditions mentioned facilitate transforming mesenchymal cells to osteoblasts, an increase in angiogenesis, withcapillary vessels invading the fibrous tissue (Molina and Ortiz-Monasterio 1995; Wangerin and Gropp, 1994).

During osteogenesis by bone distraction two mechanisms take place: endoconjunctive and endocartilaginous bone formation. They occur depending on the distraction rate and the vascular structure (Ilizarov, 1989b; Kessler *et al.*, 2004). In this study, a distraction rate of 0.5 mm per day lead to endochondral ossification, while bone fragments with calluses distracted at 1 mm per day revealed direct ossification in the matrix formed by parallel collagen fibers between the osteotomized bone fragments.

Conclusions

Bone formation by bone distraction occurs by two simultaneous mechanisms: endochondral and endoconjunctive osteogenesis. The predominance of one process or the other is dictated by the daily distraction rate. If distraction rate is close to 0.5 mm per day, endochondral bone formation prevails, whereas, in protocols using a daily distraction rate of 1mm, more endoconjunctive ossification can be observed. According to this study, optimal distraction rates vary between 0.5 and 1 mm per day. Distraction rates smaller than 0.5 mm/day lead to premature ossification of the callus. On the other hand, a distraction rate higher than 1mm/day generates a very high probability of nonunion of the bony fragments.

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STUDIA UBB BIOLOGIA, LVI, 1, 2011, p. 95-97

===SHORT COMMUNICATION===

EXPERIMENTAL RESEARCH OF BONE DISTRACTION *IN VIVO* ON A RAT MANDIBLE MODEL

OVIDIU MUREŞAN[1,✉]

SUMMARY. The purpose of the study is to assess the ossification mechanisms that occur during callus distraction using a lab rat model mandible. Radiological, clinical and histological assessments of ossification were made at different stages of neoosteogenesis. Two different and simultaneous types of bone formation occur suggesting the coexistence of endoconjunctive and endocondral osteogenesis in the distracted callus.

Keywords: bone distraction, osteogenesis, endochondral osteogenesis, endoconjunctive osteogenesis.

Introduction and aims

The aim of the present study was to analyze the ossification mechanisms of the distracted callus during and after bone distraction. The purpose was to better understand the nature of osteogenesis in distracted bone for further improving the use of this technique. The ultimate goal was to achieve a better success rate and to avoid all possible complications.

Material and methods

A number of 10 adult lab rats (Nordica) were included into this study. They were subjected to the same surgical protocol: under general anesthesia they underwent osteotomy of the mandible, between the second and third molar, and bone fragments were immobilized in the normal position for seven days needed for periosteal healing and fibrous callus formation.

After this latency period, all animal subjects underwent a protocol of mandibular distraction using artisanal devices with orthodontic screws. All rat mandibles were distracted at a 0.5mm per day rate for 6 days, resulting in 3mm distraction for each mandible, followed by a consolidation period.

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Lateral radiographs of the cranium of rats were taken immediately postoperative, after distraction and in different stages of bone consolidation (Fig. 1, Fig. 2). Animals were sacrificed at 2, 4, 5 and 6 weeks after distraction and mandibles were prepared for histological evaluation.

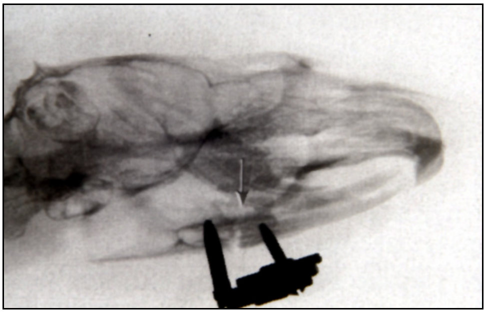

Fig. 1. Lateral cephalograph of rat before distraction.

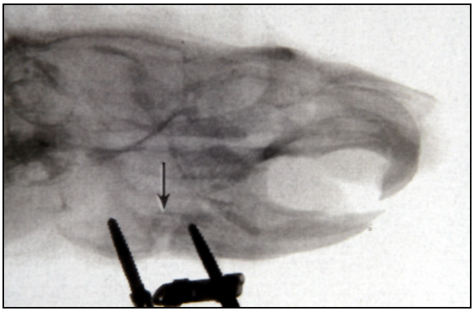

Fig. 2. Lateral cephalograph of rat after 3 mm distraction.

Results

Radiographic examination immediately after distraction revealed radio-transparency between the bone fragments and no radiological sign of ossification. Signs of mineralization occurred no sooner than 4 weeks after distraction. Microscopic examination of the distracted rat mandible fragments revealed at 2 weeks after distraction the presence of immature trabecular bone, close to the bone fragments at the peripheral callus, oriented parallel to the distraction force. Mineralization increases at 5 weeks after distraction, so it can be visible upon radiological examination. There is also a peripheralweb of immature trabecular bone that transforms gradually into mature lamellar bone by 6 weeks after distraction.

Discussions

Bone distraction is achieved by applying forces to bone fragments adjacent to a callus (Ilizarov, 1971, 1988). By gradual linear tension in the callus is subjected to constant mechanical, functional linear stress, generating an environment that is very favorable to enhancing and stimulating osteogenesis, as well at a cellular, as at a subcellular level (Asonova, 1996). The conditions mentioned facilitate transforming mesenchymal cells to osteoblasts, an increase in angiogenesis, with capillary vessels invading the fibrous tissue (Irianov, 1996).

During osteogenesis by bone distraction two mechanisms are involved: endoconjunctive and endocartilaginous bone formation. They occur depending on the distraction rate and the vascular structure (Rowe *et al*., 1998).

Conclusions

Two types of tissues were revealed in the distraction area: a fibrous, connective tissue, which slowly transforms directly into trabecular bone and a cartilaginous tissue thatis progressively invaded by capillary vesselsand is slowly replaced by mature bone. Data in the present study suggests the coexistence of two simultaneous processes of ossification in distracted callus: endochondral and endoconjunctive osteogenesis.

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STUDIA UBB BIOLOGIA, LVI, 1, 2011, p. 99-105

MICROBIAL ACTIVITY IN CAVES FROM PĂDUREA CRAIULUI MOUNTAINS (NW ROMANIA)

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MIGNON SEVERUS ŞANDOR[3] and VASILE MUNTEAN[2]

SUMMARY. Microorganisms stand at the basis of every food web in subsurface environments. We report here the presence and abundance of air and soil microorganisms isolated from three caves in Pădurea Craiului Mountains (NW Romania). We determined the density of aerobic heterotrophic bacteria and various microbial physiological groups (*i.e.* iron reducing bacteria, ammonifying bacteria, denitrifying bacteria and air staphylococci) in air and soil samples obtained from the three caves. The largest density of aerobic heterotrophic bacteria was detected in the atmosphere of Peştera de la Vadu Crişului, a cave most visited by the tourists, and the smallest in that of Peştera cu Apă din Valea Leşului, a cave that is rather inaccessible to regular tourism. The entrance zone of all three caves contained more air microorganisms then the median and profound zone of these caves. Iron-reducing bacteria, ammonifying and denitrifying bacteria were present in the atmosphere of all three caves. No staphylococci were detected in the air of the three caves. The soil microorganisms prevailed in large number in Peştera de la Vadu Crişului, while their smallest density was registered in Peştera cu Apă din Valea Leşului. The ammonifying and denitrifying bacteria dominated the soil microbial community in all three caves. The density of aerobic heterotrophic bacteria was largest in Peştera de la Vadu Crişului, while this cave contained the smallest abundance of iron-reducing bacteria. The largest density of iron-reducing bacteria was instead registered in Peştera cu Apă din Valea Leşului.

Keywords: air and soil microorganisms, cave, Pădurea Craiului Mountains.

Introduction

Microorganisms are everywhere. In cave environments, microorganisms can grow on various organic substrates, or they can develop chemolithotrophically on different inorganic compounds (Barton and Jurado, 2007; Mulec, 2008). The

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presence and diversity of microorganisms in caves can be suspected visually *in situ* as they may appear under various shapes and forms, such as dots on surfaces, unusual coloration, precipitates, corrosion residues, or biofilms (Barton, 2006). In caves, microorganisms contribute to deterioration of prehistoric wall paintings (Bastian *et al.*, 2010; Groth *et al.*, 1999; Schabereiter-Gurtner *et al.*, 2002; Schabereiter-Gurtner *et al.*, 2004); they help in the formation of caves and cave speleothemes by various means, such as indirect production of sulfuric acid (Barton and Luiszer, 2005; Macalady *et al.*, 2007); they can conduct erosion of the limestone (Cunningham *et al.*, 1995), but also calcification and mediation of crystal growth (Jones, 2001; Legatzki *et al.*, 2011). Microorganisms assist in the formation of minerals (Canaveras *et al.*, 2001, Canaveras *et al.*, 2006, Northup and Lavoie, 2001) and moonmilk (Canaveras *et al.*, 1999; Rooney *et al.*, 2010; Portillo and Gonzales, 2011), while minerals of various types select for the growth of certain types of microorganisms (Boyd *et al.*, 2007).

Due to absence of light and the obvious lack of photosynthesizing primary producers, microorganisms and dead organic materials stand at the basis of all subsurface ecosystems. In subsurface environments, microorganisms are responsible for the decomposition of organic materials transported from the surface by various means, such as the percolating water, bats, different rodents and other animals or humans visiting caves for different purposes. Besides the decomposition functions, microorganisms represent the food for various invertebrates (e.g. beetles, isopods, myriapods, springtails or mites) inhabiting subsurface habitats.

In this research, we isolated and determined the density of various types of microorganisms present in air and soil samples obtained from three caves in Pădurea Craiului Mountains (NW Romania), previously selected for a survey on groundwater microorganisms originating from the epikarst groundwater (Brad *et al.*, 2009).

Material and Methods

Research area location and sampling. Air and soil microorganisms were sampled from three caves located in Pădurea Craiului Mountains (NW Transylvania, Romania). The location of these caves (i.e. Peştera de la Vadu Crişului, Peştera Ungurului and Peştera cu Apă din Valea Leşului) was presented previously (Brad *et al.*, 2009).

Air microflora. The medium used for quantification of air and soil *aerobic heterotrophic bacteria* in the prospected caves, was identical to that used previously for detecting this type of microorganisms in water samples obtained from the same caves (Brad *et al.*, 2009). In each cave, three Petri dishes containing this growth medium were placed in all three sampling locations (the entrance zone, median zone and profound zone). The Petri dishes were opened and exposed to cave environment for 30 minutes. The dishes were then closed, sealed with parafilm, and transported to the laboratory, where they were incubated in the dark for 7 days at 30˚C. After the incubation, the bacterial colonies were counted and the density of aerobic heterotrophic microorganisms was expressed as CFU/m^3 (colony forming units/m^3) according to the following formula (Stryjakowska-Sekulska et al, 2007):

$$CFU/m^3 = \frac{A \times 10000}{P \times T \times 0.2}$$

where A is the number of colonies in a Petri dish, P is the surface area of the Petri dish in cm^2, and T is the time of exposure to cave environment in minutes. The reported values represent the average number of colonies in the three Petri dishes exposed in each sampling location.

The media used for isolation and growth of the various *physiological bacterial groups* (i.e. *Fe-reducing bacteria*, *ammonifying bacteria* and *denitrifying bacteria*) were identical to those used before (Brad *et al.*, 2009) for the identification of these types of microorganisms in water samples obtained from the same caves. For each cave, ten colonies from the Petri dishes with all aerobic bacteria were used to inoculate ten test tubes containing selective media for each type of physiological group.

Staphylococci. For detection and isolation of presumptive staphylococci, ten test tubes containing Chapman medium (Chapman, 1945) were inoculated with bacteria obtained from ten different colonies for each sample and cave. Chapman medium had a pH of 7.5 and contained (in g/l) meat extract (1), peptone (10), mannitol (10), NaCl (75), phenol red (0.025) and agar (15). For each cave, ten test tubes containing this medium were inoculated with cells obtained from the Petri dishes.

Soil microflora. Soil samples were collected aseptically from the median zone of all three caves. These samples were kept in the dark at 4°C and cultivation of microorganisms was initiated 24 h of sampling. In the laboratory, 1 g of fresh soil from each sample (cave) was mixed with 100 ml of growth medium. This medium was similar to that used for isolation of air microorganism except that the agar was not added. Serial 10-fold dilutions were performed until 10^{-10} dilution, and the cultures were incubated 7 days in the dark at 30°C. After incubation, the most probable number (MPN) of aerobic heterotrophic microorganisms present in the there caves soil was estimated according to the methods presented by Lorch *et al.* (1995). The same approach was used to estimate the density of several microbial physiological groups (i.e. Fe-reducing bacteria, ammonifying bacteria, denitrifying bacteria).

Results and discussion

Air microorganisms. The largest density of aerobic heterotrophic bacteria was registered in Peştera de la Vadu Crişului for all three zones of the cave, compared to density of this type of microorganisms in the atmosphere of the other two caves (Fig. 1).

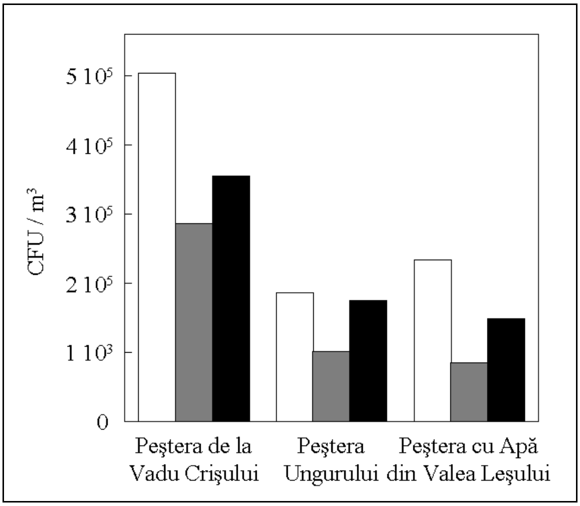

Fig. 1. Density of aerobic heterotrophic bacteria estimated as CFU per 1 m3 of air in the three prospected caves. White bars represent the density of microorganisms in the atmosphere of the entrance zones of the caves, grey bars the density of microbes in the median zones, and black bars are the densities of microbes in the profound zones of the caves

In comparison to the entrance and the profound zones, the median zone had the lowest density of air microorganisms for all three caves. The highest microbial density was registered in the atmosphere of the entrance zone of all three caves. The entrance zone of caves is normally influenced largely by the surface changing environmental conditions. This zone may contain common biotic elements with the surface, trogloxene organisms that can be only accidentally found in caves, or enter the entrance zone of caves in search for shelter (Decu and Ginet, 1971). The air currents that ventilate the entrance zone of caves can also introduce here microorganisms from the surface. We observed that the density of these microorganisms reduces with the distance from the surface towards the median zone, and increases slightly in the profound zone in all three caves (Fig. 1).

Iron-reducing bacteria, ammonifying bacteria and denitrifying bacteria were present in the atmosphere of all three caves, in all sampling locations. After incubation, no staphylococci-like growth was detected in all three caves. The presence of staphylococci, in relative low numbers, in another cave in Romania (Borda *et al.*, 2004), was related to the nursing activities of bats present in this cave in large densities during summer. According to our observations, bats only form larger colonies in Peştera cu Apă din Valea Leşului, but only hibernating colonies during winter, where bats activity is heavily reduced. The density of bats during summer, as well as the density of bats in the other two caves (i.e. Peştera de la Vadu Crişului and Peştera Ungurului) during all seasons, was always reduced to solitary individuals or very small colonies (data not shown), so that the presence of bats activity-related staphylococci is not likely.

Soil microorganisms. As already shown in figure 2, the largest density of microorganisms was detected in Peştera de la Vadu Crişului. It has been repeatedly demonstrated how show caves contain a larger density of microorganisms, which rely on a larger input of organic substrates, in comparison to more oligotrophic pristine caves (Groth and Saiz-Jimenez, 1999; Ikner *et al.*, 2006). Therefore, we related here the larger density of microorganisms, in both air and soil, obtained in Peştera de la Vadu Crişului to a larger afflux of tourists, as this cave contains common tourist-like infrastructure, such as concrete pathways and stainless steel handrails.

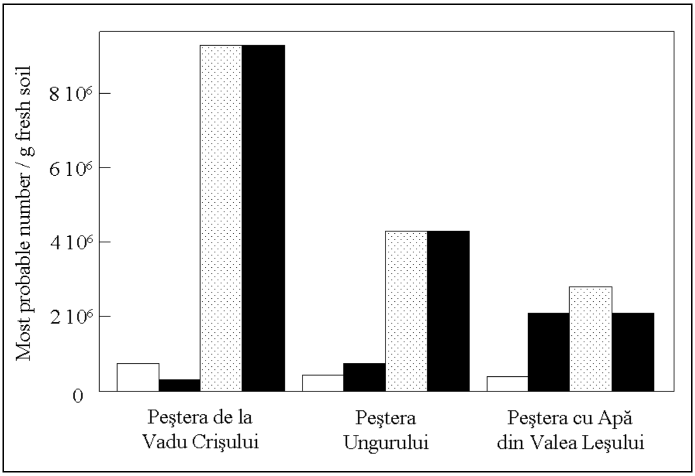

Fig. 2. Density of aerobic heterotrophic bacteria (white bars), Fe-reducing bacteria (grey bars), ammonifying bacteria (dotted bars) and denitrifying bacteria (black bars) estimated as most probable number (MPN) of microorganisms in 1 gram of cave soil

The most abundant of all determined physiological groups of microorganisms were those involved in the nitrogen cycle (i.e. the ammonifying and denitrifying bacteria), also with the largest density in Peştera de la Vadu Crişului and the smallest in Peştera cu Apă din Valea Leşului. The density of denitrifying bacteria obtained in our study was comparable to that obtained in similar studies (Arroyo *et al.*, 1997). The large number of these microorganisms obtained in all three caves relate most probably to the presence in these soils of larger amounts of organic matter that is further subjected to ammonification, nitrification and finally denitrification. The density of ammonifying bacteria had comparable values with that of denitrifying bacteria in all three caves.

Like for the nitrogen cycle-related bacteria, the largest density of aerobic heterotrophic bacteria was detected in Peştera de la Vadu Crişului, and the smallest in Peştera cu Apă din Valea Leşului. In contrast, the largest density of iron-reducing bacteria was registered in Peştera cu Apă din Valea Leşului, while the smallest density of this type of microorganisms was detected in the soil of Peştera de la Vadu Crişului.

In comparison to the density of soil microorganisms obtained during this study, the microorganisms isolated from the percolating water in the three caves were most abundant in Peştera cu Apă din Valea Leşului, while the lowest microbial density was obtained for the water dripping in Peştera de la Vadu Crişului (Brad *et al.*, 2009). The density of the different microbial groups in the soil was in general larger compared to that of water microorganism isolated from the same caves.

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