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Original picture on front cover: *Astragalus tarchankuticus*

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THE DIVERSITY OF APIONIDAE SPECIES (COLEOPTERA: CURCULIONOIDEA) FROM THE SURROUNDINGS OF CLUJ-NAPOCA, ROMANIA

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SUMMARY: This paper presents a study of Apionidae (Coleoptera: Curculionoidea) that inhabit the surroundings of Cluj-Napoca, therefore providing additional information on the distribution of these insects in Romania. In order to asses species diversity, we have sampled biological material from April to June 2008. The sampling stations included two anthropic (orchards, pastures) and three non-anthropic (deciduous and coniferous forest, meadow) habitats. A number of 18 species belonging to 8 genera were recorded. Among these species, we noticed the presence of two rare ones not reported previously in Cluj-Napoca, namely *Eutrichapion (Psilocalymma) facetum* (Gyllenhal, 1859) and *Eutrichapion (Psilocalymma) punctiger* (Paykull, 1792). The identified species belong to four zoogeographical elements.

Keywords: Apionidae, Bray-Curtis index, ecology, diversity, multivariate analysis (MVA).

Introduction

From a total of more than 2000 species belonging to the family Apionidae described until present, more than 500 species are reported from the entire Palearctic Region, out of which over 400 species are present in the Western Palearctic region solely (Russel, 2001). This family represents a significant proportion of the insect diversity in the ecosystems to which they belong. Some Apionidae are pests for agricultural crops while others are vectors for plant disease. Some species of this family are important as biological agents against weeds from agroecosystems. Regarding their economical importance, Russell, 2004 stated "...*Protapion* has a conflict of interest with *Homo sapiens* over their competing parasitism of *Trifolium* species".

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Recently, a list of Curculionidae from Romania has been published (Teodor and Antonie Vlad, 2007) that also encompasses the species of Apionidae family. From this paper a number of 138 Apionidae species have been reported until present in Romania.

The main goal of the paper is to asses the presence and distribution of Apionidae species in the surrounding areas of Cluj-Napoca and to provide new data for this taxon.

Material and methods

The biological material has been sampled from the surrounding areas of Cluj-Napoca in 2008, between April and June. Different typologies of anthropic and non-anthropic habitats were included in the analysis, in 7 sampling stations. The sampling stations are:

1. Hoia forest – deciduous species like oak (*Quercus petraea*), *Carpinus*, sp.
2. Baciului Gorge – deciduous and hygrophile species
3. Coruşu – meadow, near the village road, edified by herbal species and some marginal Elderberry shrubs (*Sambucus nigra*).
4. Steluţa Hill – apple orchard with some marginal bushes like *Prunus spinosa, Crataegus monogyna*, *Coryllus avellana*, *Viburnum* sp., *Cornus sp.*
5. Floreşti – orchard situated near a scots pine plantation
6. Făget-Sf. Ion – mezophilous meadow edified by Umbelifere, *Centaurea* sp., *Origanum vulgare*, etc
7. Feleac Hill – meadow and apple orchard

Sampling was carried out by sweeping the vegetation with the aid of the entomological net, as it represents probably the best method of sampling both phytophagous and aquatic beetles as well (Grootaert *et al*., 2010). The sampling stations yielded a total number of 110 individuals that were preserved in 70-80% alcohol tubes before their taxonomical identification.

Every tube was labeled with the locality, date, name of the collector, type of ecosystem and the substrate form (i.e. herbal layer, trees, bushes). Every single habitat of collection was photographed and characterized. The identification of the species was conducted in the laboratory, on the basis of morphological characters and male genitalia. A binocular magnifier and specific literature (Alonso-Zarazaga, 1990; Alonso-Zarazaga and Lyal, 1999; Behne, 1998; Dieckmann, 1977; Freude *et al*., 1981, 1983) were used for the identification. The repartition of family species on tribes was made in accordance with the present accepted nomenclature (Alonso-Zarazaga, 2011).

A multivariate analysis of community was performed on the species-per-site abundance matrix (square root transformed) using the software PRIMER 6 + PERMANOVA software package from Plymouth Marine Laboratory, UK (Clarke and Gorley, 2006). In particular, the analysis included a comparison of the 7 sampling sites based on total abundance (N), species richness (R), Shannon diversity (H') and Evenness (J). An *a priori* ordination of the sampling sites in relation to the community composition of Apionidae was obtained by computing the Bray-Curtis similarity index (Bray and Curtis 1957) and applying a basic agglomerative hierarchical cluster algorithm using the group average approach, which defines cluster proximity as the average pairwise proximity of all pairs of points from different clusters. This technique was selected as particularly recommended for a dendrogram plot based on the Bray-Curtis similarity index.

Results

We have identified a number of 18 species from 8 genera belonging to family Apionidae (Table 1).

Two rare species were reported in the investigated area:

1. Eutrichapion (Psilocalymma) facetum (Gyllenhal,1859*)*

Collected material: 1 ♀, 28.V.2008 – Hoia forest (sampling station 1).
General distribution: Europe, Western and Central Asia , Siberia, Mongolia.
In Romania: Banat. (Pălăgeşiu, 1974).
Biology and ecology: generally oligophagous on host plants like Vicia species (*Vicia cracca*, *V. sepium*, etc) (Mazur, 2002). Adults are active from May to August.

2. Eutrichapion (Psilocalymma) punctiger (Paykull,1792)

Collected material: 1 ♂, 2 ♀, 29. V. 2008 – Feleac Hill (sampling station 7).
General distribution: Europe, from Eastern-Western Kazakhstan, Western Asia, Northern Africa.
In Romania: - Transylvania (Seidlitz, 1891; Holdhaus & Deubel, 1910; Petri, 1912, 1925/'26; Teodoreanu, 1986a; Kocs & Podlussány, 1997, 1999); Muntenia (Greater Wallachia) (Jaquet, 1899b; Fleck, 1905; Montandon, 1908; Wagner, 1910a); Dobrogea (Montandon, 1908); Banat (Pălăgeşiu, 1970, 1971, 1974, 1986).
Biology and ecology: generally oligophagous on different species of Vicia (Mazur, 2002). The adults are active from the end of May until the beginning of August.

Table 1. Apionidae species recorded from the surrounding areas of Cluj-Napoca between April-June 2008.

Clasification/Species	Date	N	St.	Distribution in Romania	General
Curculionoidea **Apionidae Apioninae Exapiini**					
Exapion (s. str.) compactum (Desbrochers des Loges, 1888)	29.V	1	7	Tr, Bn	euro
Kalcapiini					
Taeniapion urticarium (Herbst, 1784)	28.V	2	1	Tr, Bn, M, Mt, Db	euro-w-c-as
		1	3		
Oxystomatini					
Catapiina					
Catapion seniculus (W. Kirby, 1808) *	28.V	1	1	Tr, Bn, Mm, M, Mt, Db	pal
		1	4		
	29.V	1	5		
		1	7		
Oxystomatina					
Eutrichapion (s. str.) viciae (Paykull,1800) *	28.V	1	1	Tr, Ot, Mt, M, Db	pal
	3.VI	7	6		
Eutrichapion (Phalacrolobus) melancholicum (Wencker 1864)	28.V	2	4	Tr, M, Mm, Bn	euro-w-c-as
*Eutrichapion (Psilocalymma) facetum (*Gyllenhal,1859*)* **rs**	28.V	1	1	Bn	euro-w-c-as
Eutrichapion (Psilocalymma) punctiger (Paykull,1792) **rs**	29.V	3	7	Tr, M, Mt, Db, Bn	euro-w-c-as
Hemitrichapion (Dimesomyops) pavidum (Germar, 1817)	25.V	1	6	Tr, Bn, Mm, M, Mt	pal
	29.V	3	6		
Synapiina					
Ischnopterapion (Chlorapion) virens (Herbst, 1797) *	3.VI	2	6	Tr, M, Db, Mm, Bn	pal
Ischnopterapion (s. str.) *loti* (W. Kirby, 1808) *	3.VI	10	6	Tr, Bn, Ot, Db	pal
	28.V	1	1		
	29.V	2	5		
		2	7		
Trychapiina					
Trichopterapion holosericeum (Gyllenhal, 1833)	28.V	1	1	Tr, M, Mt, Db, Bn	euro-w-c-as
	29.V	1	7		
	3.VI	1	6		
Piezotrachelini					
Protapion apricans (Herbst, 1797)*	23.IV	1	6	Tr, Mm, M, Bn, Ot, Mt, Db	pal
	28.V	3	1		
		2	2		
		1	4		

Clasification/Species	Date	N	St.	Distribution in Romania	General
	29.V	2	5		
	1. VI	1	6		
	2. VI	1	6		
	3.VI	1	6		
Protapion assimile (W. Kirby, 1808) *	29.V	6	5	Tr, Mm, M, Mt, Bn	pal
		3	7		
	3.VI	7	6		
Protapion filirostre (W. Kirby, 1808) *	28.V	1	1	Tr, Bn, M, Mt, Db	eurosib
	29.V	1	7		
Protapion fulvipes (Fourcroy, 1785)	3.VI	4	6	Tr, Mm, M, Bn, Ot, Mt, Db	pal
	28.V	4	1		
		1	2		
		11	4		
	29.V	3	5		
		1	7		
Protapion nigritarse (W. Kirby, 1808) *	28.V	2	1	Tr, M, Mt, Db, Bn	euro-w-c-as
		2	4		
Protapion ruficrus (Germar, 1817)	28.V	1	1	Tr, Mm, M, Mt, Bn	euro
Protapion trifolii (Linne, 1768)	28.V	1	3	Tr, Bn, Mm, M, Mt, Db	pal
		1	4		
	29.V	3	5		

Abreviations:

N = number of individuals/ sampling station, St. = see description of sampling stations, * = species reported previous to our research (see tab.1), rs = rare species

Distribution in Romania: **Bn** = Banat, **Cr** = Crisana, **Db** = Dobrudja, **M** = Moldavia, **Mm** = Maramures, **Mt** = Muntenia, **Ot** = Oltenia, **Tr** = Transylvania (bibliographical data presented in Teodor and Vlad Antonie, 2007).

General: **euro** = European species, **eurosib** = Eurosiberian species, **euro-w-c-as** = European, western and central Asian species, **pal** = Palearctic spread species.

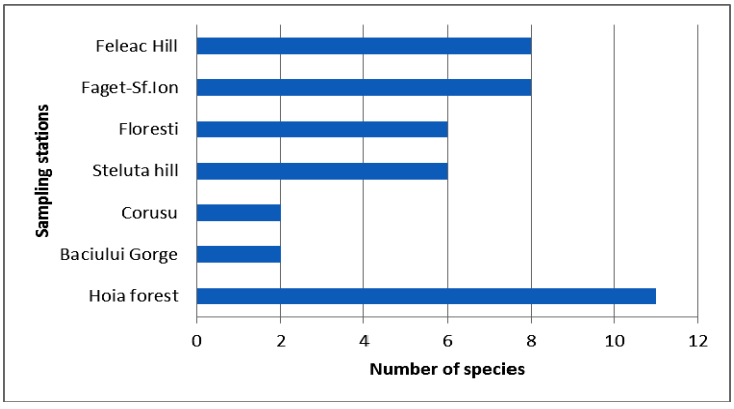

Figure 1. Numeric distribution of Apionidae species by sampling stations

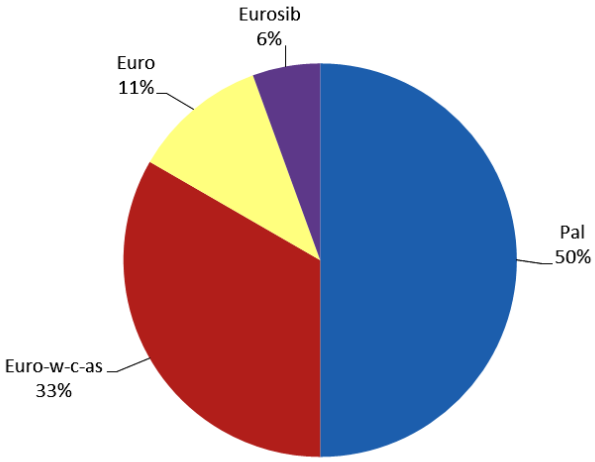

Figure 2. Zoogeographical spectrum of Apionidae from the surroundings of Cluj-Napoca

Hoia forest and Feleac Hill are the most relevant habitat for Apionidae diversity (Table 2). In fact, on this site the Shannon diversity (H') and Evenness (J') are the highest as compared to the other sampling sites, particularly the Hoia forest, where the species richness is the highest (S=11).

Table 2. Ecological indices of Apionidae species in the 7 sampling station of Cluj-Napoca
S = Species Richness; N = total abundance; H'(loge) = Shannon diversity index;
J' = Evennes idex

Sampling stations	**S**	**N**	**H'(loge)**	**J'**
Hoia forest (deciduous)	11	18	2,245	0,9363
Baciului Gorge (meadow)	2	3	0,6365	0,9183
Corusu (meadow)	2	2	0,6931	1
Steluta hill (orchard)	6	18	1,271	0,7093
Floresti (orchard + scots pine plantation)	6	17	1,65	0,9209
Sf. Ion (meadow)	8	39	1,913	0,9197
Feleac hill (meadow + orchard)	8	13	1,951	0,9384

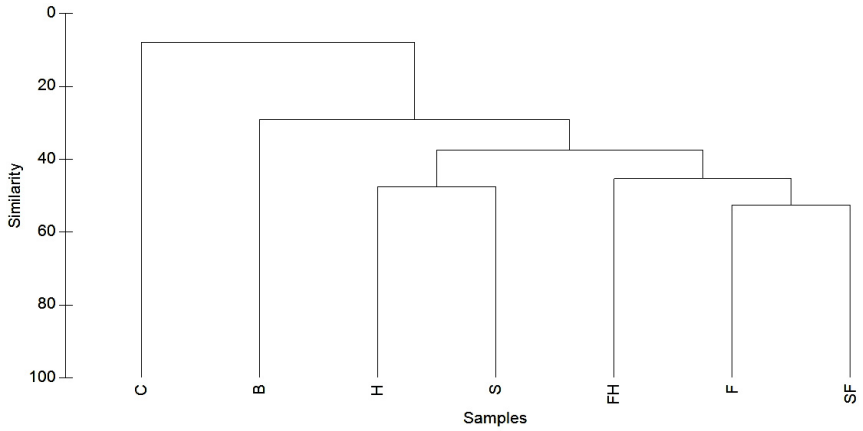

Figure 3. Dendrogram plot based on the Bray-Curtis similarity index

The cluster analysis (Fig. 3) shows that two groups of stations (H = Hoia forest; S = Steluta hill and Baciului Gorge; F = Floresti and Feleac Hill) have similar species composition, clustering at about 40% similarity. To the contrary, especially Corusu sampling station and Sf. Ion appear different in community structure, being separated at 10%-30% similarity. Thus, the dendrogram identifies a general common pattern of Apionidae species composition in sampling stations characterized by high trees, but not in the ones characterized by meadows, that in fact do not cluster together.

Discussions

In the area of Cluj-Napoca, before the present study, a total of 20 species of Apionidae (Table 3) have been reported in four publications (Petri 1912; Perju *et al*., 1986, 2000; Stugren *et al*., 1969).

This study, compared to other investigations, shows a relatively higher number of identified species of Apionidae family (18 species) in the studied area. Petri, 1912 describes 3 species of the same family. An examined apple tree orchard situated near the city of Cluj (Stugren et al., 1969) revealed a number of 5 species of Apionidae. A study involving the seed feeding in Curculionidae family (Perju *et al.*, 1986) mentions 11 species of Apionidae. While investigating the entomofauna of fodder plants (Perju *et al*., 2000) 3 species of this family were reported.

By comparing the identified species with those reported previously, we found 7 new records from the surrounding areas of Cluj-Napoca: *Exapion (s.str.) compactum* (Desbrochers des Loges, 1888); *Taeniapion urticarium* (Herbst, 1784); *Eutrichapion (Phalacrolobus) melancholicum* (Wencker, 1864); *Eutrichapion (Psilocalymma) facetum* (Gyllenhal, 1859); *Eutrichapion (Psilocalymma) punctiger* (Paykull, 1792);

Hemitrichapion (Dimesomyops) pavidum (Germar, 1817); *Trichopterapion holosericeum* (Gyllenhal, 1833); *Protapion fulvipes* (Fourcroy, 1785); *Protapion ruficrus* (Germar, 1817) and *Protapion trifolii* (Linne, 1768).

Table 3. Apionidae species identified previous to our research from the Cluj-Napoca area

Nr.	Clasification/species	Author and year of report
	Superfamily Curculionoidea	
	Family Apionidae	
	Subfamily Apioninae	
1	*Apion frumentarium* (Linné, 1758)	Petri, 1912
2	*Rhopalapion longirostre* (Oliver, 1807)	Petri, 1912
3	*Oxystoma cerdo* (Gerstäcker, 1854)	Petri, 1912
4	*Protapion apricans* (Herbst, 1797)	Perju *et al*., 1986 Stugren *et al*., 1969
5	*Protapion filirostre* (Kirby, 1808)	Perju *et al*., 2000
6	*Protapion interjectum* (Desbrochers des Loges, 1895)	Perju *et al*., 2000
7	*Protapion schoenherri* (Boheman, 1839)	Perju *et al*., 2000
8	*Protapion assimile* (Kirby, 1808)	Perju *et al*., 1986
9	*Protapion varipes* (Germar, 1817)	Perju *et al*., 1986
10	*Protapion nigritarse* (Kirby, 1808)	Perju *et al*., 1986 Stugren *et al*., 1969
11	*Ischnopterapion (s.str.) loti* (Kirby, 1808)	Perju *et al*., 1986
12	*Ischnopterapion (Chlorapion) virens (*Herbst, 1797*)*	Stugren *et al*., 1969
13	*Stenopterapion tenue* (Kirby, 1808)	Stugren *et al*., 1969
14	*Stenopterapion meliloti* (Kirby, 1808)	Perju *et al*., 1986
15	*Catapion seniculus* (Kirby, 1808)	Stugren *et al*., 1969
16	*Oxystoma pomonae* (Fabricius, 1798)	Perju *et al*., 1986
17	*Oxystoma craccae* (Linne, 1767)	Perju *et al*., 1986
18	*Eutrichapion (s. str.) viciae* (Paykull, 1800)	Perju *et al*., 1986
19	*Eutrichapion (s. str.) ervi* (Kirby, 1808)	Perju *et al*., 1986
20	*Pseudoperapion brevirostre* (Herbst, 1797)	Perju *et al*., 1986

The majority of the species have a large distribution in Romania (Table 1), while only a few species have a narrower area of distribution. *Exapion (s.str.) compactum* (Desbrochers des Loges, 1888) is found only in Transylvania and Banat. *Eutrichapion (Psilocalymma) facetum* (Gyllenhal) has been reported only from localities in Banat (Pălăgeşiu, 1974) until now, while its presence in Transylvania is reported here for the first time. The trophical spectrum of identified species comprises oligophagous and monophagous species that are strongly associated with meadows and pastures (Podlussany et al., 2001; Mazur, 2002). Among these trophical categories, oligophagous

species dominate the range while only three species are restricted to monophagy, namely *Taeniapion urticarium, Hemitrichapion (Dimesomyops) pavidum and Trichopterapion holosericeum.*

Zoogeographically, the Apionidae fauna from Cluj-Napoca surrounding areas shows a relatively high diversity with four elements (Fig. 2). The predominance of Palearctic species (9 species, 50%) can easily be observed, closely followed by the euro-west-central-asian (6 species, 33%) and european ones (2 species, 11 %). The lowest percentage consists of the species with a eurosiberian distribution (1 species, 6%).

The reported species have a different distribution in the studied habitats (Fig.1), the greatest number of species being present in the Hoia forest (11 species) followed closely by hygrophilous meadow from Făget – Sf. Ion and the apple orchard from Feleac Hill (8 species). The sampling stations from Floreşti and Steluţa Hill have a relatively low number of species (both with 6 species). The lowest number was found in Coruşu and Baciului Gorge (both with 2 species).

The multivariate analysis of community structure of apionidae shows the fact that while sampling stations with high trees have a similar composition in apionidae species, each meadow station presents a low diversity community characterized by different species.

The diversity encountered in Hoia forest may be attributed to the fact that this area is situated at the edge of the forest, where the wooden species meet the ones from the meadows in the ecotone zone. The diversity of the ecotone was also reported for other edaphic coleoptera from Sic-Pastaraia (Transylvanian Plain) (Nitu, 2007). Oppositely, the lowest diversity from Corusu and Baciului Gorge may be explained by the low height of the herbaceous layer which cannot support a very high number of species since generally increased levels of apionidae species diversity are associated with taller vegetative layers (Fenner and Palmer, 1998).

Future studies and greater number of samples are needed in order to have a more comprehensive assessment of the whole Apionidae community composition.

Conclusions

- A number of 18 species from 8 genera of Apionidae family have been identified from the surrounding areas of Cluj-Napoca.
- Two rare species were reported in the studied area: *Eutrichapion (Psilocalymma) facetum* (Gyllenhal, 1859) which is reported for the first time in Transylvania, and *Eutrichapion (Psilocalymma) punctiger* (Paykull, 1792).
- Palearctic species were predominant, followed by the euro-west-central-asian and european ones.

- The dominant species was *Protapion fulvipes* (F.) followed by Protapion *assimile* (K.) and *Ischnopterapion (s. str.) loti* (K.)
- The greatest number of species was reported in Hoia forest (11 species), in contrast to Coruşu and Baciului Gorge, where only two species were identified.

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STUDIA UBB BIOLOGIA, LVII, 1, 2012 (p. 15-20)

SPONTANEUOS VEGETATION DEVELOPMENT ON MINING WASTE DUMPS FROM ROŞIA MONTANĂ (ROMÂNIA)

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SUMMARY: The general trends of the spontaneous vegetation development on the mining waste dumps from Roşia Montană were investigated and the relationships between the vegetation cover, waste dump age and some of its geomorphological characteristics were studied. The analysis conducted on the 60 years old waste dump identified 53 mining spoils patches that are not covered yet with spontaneous vegetation. In addition to the mining spoil patches, three vegetation patch types were distinguished within the study area: grasslands, heathlands and woodlands. At the landscape level, in all patch types we identified 267 plant species but on the oldest mining spoils patches we only find 61 species represented by 1-10 individuals. Regarding the geomorphological characteristics of the oldest dump, we find that shadier slopes have a higher vegetation cover than sunny slopes. Also the vegetation cover of exposed spoils decreases with increasing distance to semi-natural grassland patches.

Keywords: mining waste dump, Roşia Montană, spontaneous vegetation succession.

Introduction

The intensive mining of gold has always affected the overlying ecosystems, the land-use potential, and the attractiveness of landscapes. Furthermore, mining leaves behind damage stretching over large areas. It is therefore an important task to understand the natural processes related to succession for cost-effective, successful restoration of such areas (Cristea *et al.*, 1990).

The present research has the following objectives:

(1) to study the spontaneous vegetation development on the mining waste dumps from Roşia Montană (Alba County), in order to identify measures that could lead to an effective economical and ecological restoration;

(2) to reveal the relationships between the vegetation cover, waste dump age and some of its geomorphological characteristics.

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Beyond these purely scientific purposes of our work, we wanted to bring forward some evidence to support the protection of the landscape according to the European Landscape Convention (Florence, October 2002), which was ratified by Romania through the Law no. 451/July 8, 2002.

Materials and Methods

The field investigations were carried out in 2007-2010 on abandoned gold mining waste dumps placed around the existing opencast pits – Cetate and Cârnic (Fig. 1) from Roşia Montană (locality situated in the Apuseni Mountains, in NW part of the Alba County).

Our study focused only on waste dumps that have resulted from the open cast pits without analyzing the status of the other existing tailing mine facilities. Both types of dumps are in fact huge anthropogenic landscape formations, which have a visible negative effect on the semi-natural landscape of this region.

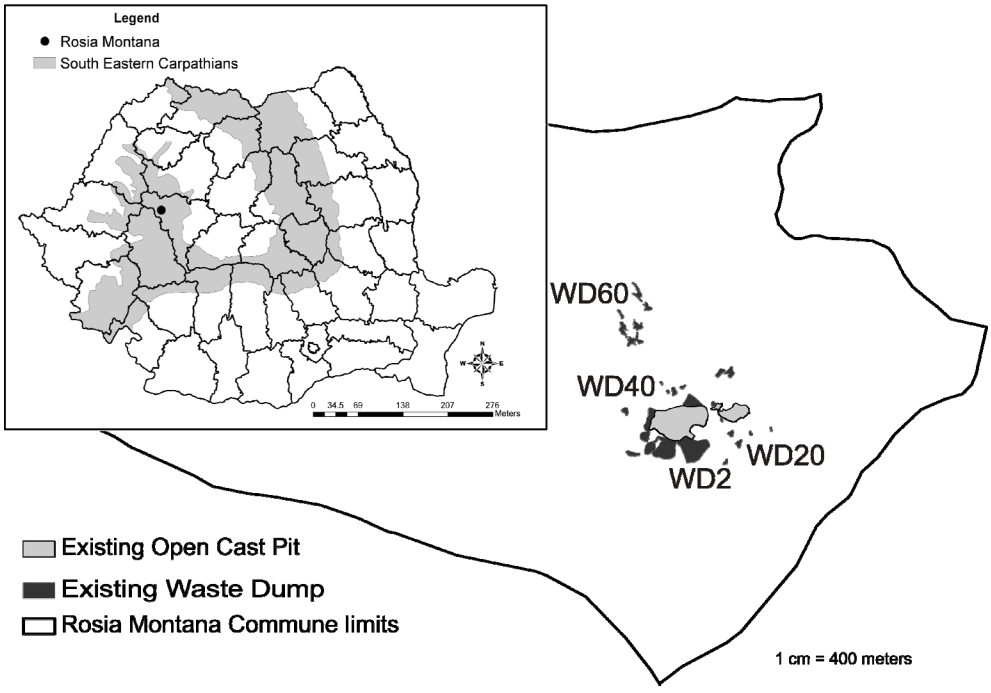

Figure 1. The study sites from Roşia Montană (abbreviations in text)

The following waste dumps, which had similar environmental conditions and management histories but differed in the time since the mining activities ceased, were selected for our research: WD2, ~ 2 yrs old (Valea Verde, Hop and Rakosi Waste Dumps); WD20, 20-25 yrs old (Cârnicel and Piatra Corbului Waste Dumps); WD40, 38-45 yrs old (Iuliana, Aurora and Afiniş Waste Dumps); and WD60 ~ 60 yrs old (Orlea Waste Dump). The area surrounding these dumps is used as meadow or pastureland, with some woodlots included. The potential natural vegetation in the study area is a forest dominated by beech - *Symphyto cordati-Fagetum* (Pop, 1976), but the forest patches next to waste dumps are composed mainly of pioneer species like *Betula pendula, Populus tremula*, *Salix caprea* and *Pinus sylvestris*. Other patches are composed of heathlands (*Calluna vulgaris, Vaccinium myrtillus* and *V. vitis-idaea*) or semi-natural grasslands dominated by *Agrostis tenuis, Festuca rubra*, *Poa pratensis* and *Deschampsia flexuosa* (Ghişa *et al*., 1970; Pop, 1976).

At each waste dump we recorded all vascular plant species within 5 randomly placed 1 m^2 quadrats. These data were used to study the spontaneous vegetation development on the mining waste dumps, especially the variation in species richness along the chronosequence of sites. The differences in species number were estimated using the Kruskal-Wallis test.

We also performed detailed floristic inventories in 5 x 5 m^2 plots on the oldest dump in order to analyse the importance of the landscape context.

We digitised all vegetation patches in ARC/INFO (ESRI, 2009) by using georeferenced topographic maps (at scale 1:5000), aerial photographs with a cell size (pixel) of 0.5 m, and additional ground information. A digital 20-m elevation model was used to determine mean slope and aspect for each plot.

Results and Discussion

The analysis conducted at lanscape level (25 ha) revealed that on the 60 yrs old waste dump there still may be as much as 53 mining spoils patches that are not covered yet with vegetation. In addition to the mining spoil patches, three vegetation patch types were distinguished within the study area, i.e. grasslands, heathlands and woodlands (Fig. 2). The area of spoil patches ranges between 25 m² and 0.8 ha. At the landscape level, in all vegetation patches we identified 267 plant species but on the oldest mining spoils patches we only find 61 species represented by 1-10 individuals.

Regarding the geomorphological characteristics of the oldest dump, we find that shadier slopes have a higher vegetation cover than sunny slopes. Also the vegetation cover of exposed spoils decreases with increasing distance to semi-natural grassland patches.

The most common vascular plant species that occurred spontaneously on the mining dumps studied were:

- WD2: *Tussilago farfara, Poa compresa, Agrostis capillaris,* and *Polygonum aviculare*;
- WD20: *Betula pendula, Pinus sylvestris, Populus tremula, Agrostis capillaris* and *Deschampsia flexuosa*;
- WD40: *Agrostis capillaris, Poa pratensis, Chamaespartium sagittale* and *Deschampsia flexuosa*;
- WD60: *Calluna vulgaris, Vaccinium vitis-idaea, V. myrtillus*, *Betula pendula* and *Populus tremula*.

Plant species with low frequencies on these waste dumps are: **WD2**: *Festuca rubra*; **WD20**: *Gymnadenia conopsea, Pulmonaria rubra, Melampyrum bihariense*; **WD40**: *Dactylorhiza sambucina, Dactylorhiza incarnata, Orchis morio, Orchis ustulata, Listera ovata*, *Melica ciliata* and *Sedum annum*; **WD60**: *Lycopodium clavatum, Quercus petraea, Gentiana cruciata* and *Platanthera bifolia.*

The pattern of species frequencies along this acidophilous site cronosequence corresponds roughly to the well-known replacement of pioneer herbs (e.g., *Tussilago farfara*) by grasses (e.g., *Poa pratensis*) and then by mid-successional shrubs (e.g., *Vaccinium vitis-idaea*).

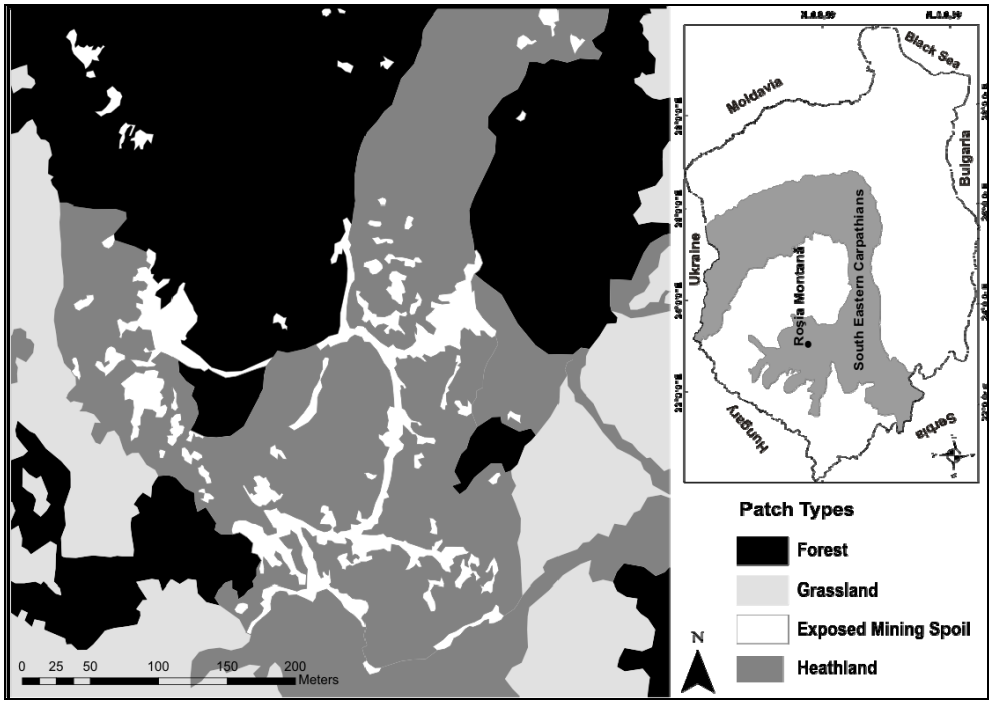

Figure 2. Distribution of vegetation patch types throughout the landscape circumscribing the Orlea mining waste dump.

Species number generally increases from the youngest waste dumps toward the older ones (fig. 3). This pattern is obviously associated with the colonization and establishment of new species after disturbance. However, we observed a decline in species richness on the oldest waste dump, which could represent a mid-successional stage when competitive exclusion occurs in plant assemblages. This outcome is consistent with the general pattern observed in terrestrial ecosystems, i.e. a decrease in species numbers as successional species are lost (Begon *et al*., 2006; Peet, 1992). However, the change in species richness during succession also depends on the environmental context (Peet, 1992). Therefore, the decline observed on the oldest waste dump might be the consequence of small disturbances that occurred before our survey time (e.g., erosion caused by water run-off).

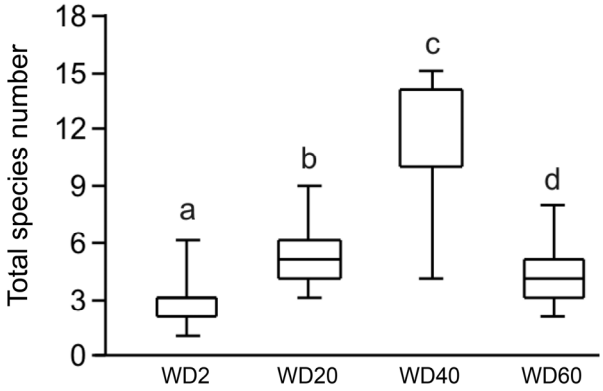

Figure 3. Changes in species number across age classes. The mid line inside the box-and-whisker represents the median (from Roman *et al*., 2009).

Conclusions

Because there is a negative relationship between the vegetation cover of exposed spoils and the distance to the nearest grassland patch, we may assume that grasslands are the main seed source for the spontaneous vegetation development. In order to restore and "accelerate" the spontaneous vegetation development on these waste dumps, we recommend increasing connectivity with grassland patches. Also, to reduce erosion it is absolutely necessary to perform binding with a layer of soil and to stabilize the slopes. Other studies conducted on spoils containing Pb and Zn impurities recommended the following steps for their ecological restoration: 1. covering with a minimum 10-cm soil layer; sowing of a complex of species (preferentially from the native flora of the region) and periodical fertilization and watering, at least during the first 3-4 years of recultivation; 2. plantation of trees and shrubs in accordance with their ecological requirements and site conditions existing on each terrace (Cristea *et al*., 1995).

The vegetation cover is higher on shady and gentle slopes, which suggests that the hydric deficit is a limiting factor for the vegetation development, besides the lack of a structured soil layer.

Even without any active measures of restoration, a steady increase in both plant species number and cover with the age of waste dumps was observed. However, spontaneous vegetation development is a very slow process on these dumps due to the extreme site conditions (i.e., high content in heavy metals, lack of structured soil and humus). Therefore, a man-assisted ecological restoration of these mining waste dumps is required, even if it involves certain investments!

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STUDIA UBB BIOLOGIA, LVII, 1, 2012 (p. 21-32)

THE EFFECT OF LIGHT STRESS ON PS II PHOTOCHEMICAL ACTIVITY AT *NOSTOC LINCKIA* AICB 421

VICTOR BERCEA[1], ADRIANA HEGEDŰS[1], NICOLAE DRAGOŞ[1,2] and COSMIN SICORA[1,3,✉]

SUMMARY: The changes in chlorophyll fluorescence during a two hour period of high light were analyzed in cyanobacterium *Nostoc linckia* AICB 421. A light intensity of 800 μmoli.m^{-2}.s^{-1} induced the decrease of Fm, Fv and maximal and effective quantum yield. In conditions of exposure to 1500 μmoli.m^{-2}.s^{-1}, F_0, F_m, Fv and maximal and effective quantum yield decreased also, relative to control. A 2100 μmoli.m^{-2}.s^{-1} light intensity increased the F_0, Fm and Fv and the quantum yield values lowered. Photochemical coefficients maintained maximal values. Quantum yield of non-regulated energy dissipation Y(NO) was enhanced. Changes in chlorophyll fluorescence were caused by structural changes in the antenna and in the PSII reaction center, due to light stress.

Keywords: minimal and maximal fluorescence, maximal and effective quantum yield, quantum yield of nonregulated energy dissipation, coefficient of photochemical quenching, phycobilisomes.

Introduction

Cyanobacteria are the most versatile prokaryotic organisms, which live in various terrestrial and aquatic ecosystems (Paerl and Huisman, 2009). These organisms contained thylakoids similar with multi-protein complexes which use the light energy to co-ordinate the electron transfer, consequently producing the NADPH and ATP. The key factors involved in light driven water oxidation and primary charge separation are located in photosystem II. The main difference between the cyanobacteria and chloroplasts is light harvesting antenna associated with PS II.

In cyanobacteria, this structure contains several phycobilin pigments which are organized in macromolecular complexes attached to the thylakoids surface, named phycobilisomes. Unlike the cyanobacteria, in plants, the antenna are formed by proteins imbedded in the thylakoid membrane, linked to a and b chlorophylls. Both in plants

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and cyanobacteria, the cell content of light harvesting complexes is regulated by light, thus the phycobilisomes content changes rapidly and as a results to the presence of other factors, like nitrogen and CO_2, also (Grossman *et al*., 1993; Reuter and Müller, 1993; Tandeau de Marsan and Houmard, 1993).

The loss of photosynthetic activity due to the absorption of the excessive light, process called photoinhibition is common in plants and cyanobacteria (Samuelsson *et al*., 1985; 1987; Lönneborg *et al*., 1988; Krupa *et al*., 1990). The photoinhibition takes place in conditions when the harvested light exceeds the dissipated energy, either as a result of the exposure to high light or moderate light if an excessive light harvesting antenna is present, or if the low temperature limits the energy dissipation (Huner *et al*., 1993). The most sensitive region to photoinhibition is the PS II reaction center, thus *in vivo* the key reactions become inactive. *In vitro*, photoinhibition is produced after the primary charge separation if the electron donation at the oxidized reaction center of PS II is blocked or through over-reduction the primary electron acceptor quinone (Q_A) located at the edge of PS II (De Las Rivas *et al*., 1992). Both types of inhibition are the result of D_1 protein degradation (Shipton and Barber, 1991; Vass *et al*., 1992).

The results of the present study point out the PS II photosystem activity measured by chlorophyll fluorescence in *Nostoc linckia* AICB 421 which was exposed to long periods of various light intensities.

Materials and methods

The cyanobacteria *Nostoc linckia* AICB 421 (Nostocales) was cultivated at room temperature on GZ medium, using a medium light intensity of 26 $\mu mol.m^{-2}.s^{-1}$, in air-lift conditions. The growth period was set to 8 days. The cells found in exponential growth rate were subjected to high light intensities of 800, 1500 and 2100 $\mu mol.m^{-2}.s^{-1}$, for a two hour period. The light source was an incandescent lamp FHI-5000 W and the light intensity was measured with Quantum Sensor QSPAR Hansatech.

The effect of light exposure was measured during 15, 30, 45, 60, 75, 90, 105 and 120 min. During the recovery period, 120 min. from ceasing the light stress, specific fluorescence measurements were taken. The photosystem II activity was evaluated by chlorophyll fluorescence parameters of light exposed probes, using a Waltz Dual-100 fluorometer.

The assimilatory pigments (chlorophyll a and carotenoids) were extracted in acetone and spectrophotometrically estimated based on specific absorption coefficients (Amon, 1949, Lichtenthaler and Wellburn, 1983), thus their identification was done based on the maximal absorption peak using a Jasco V-630 spectrophotometer. Phycobiliproteins were estimated according to Gantt and Lipschultz (1974). The results were expressed in mg/l cellular suspension.

Results and discussions

The cell culture of *Nostoc linckia* AICB 421 showed an optical density (OD_{680}) of 0.61. The *in vivo* absorption spectra of the cell suspension emphasized the blue spectra where the absorption peaks of carotenoids (482 nm) and chlorophyll a (439 nm) are located and the red spectra of phycobilins (621 nm) and chlorophyll a (681 nm). The absorption peak of (572 nm) induced by large amounts of phycoerythrin is visible (fig. 1).

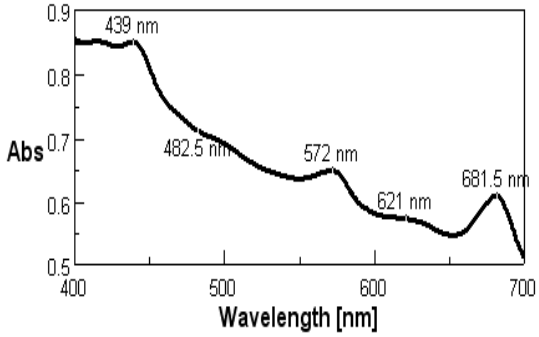

Figure 1. The *in vivo* absorption spectra of *Nostoc linckia* AICB 421 cell suspension, cultivated in standard conditions.

In cyanobacteria, the components of the photosynthetic apparatus include chlorophyll a (665 nm) which joins various forms of carotenoids and phycobiliproteins forming the light harvesting antenna. The pigments concentration was measured in the initial moment, prior to light stress exposure (control conditions).

Table 1. Includes the quantities of the assimilatory pigments (mg/l)

Pigments (mg/l)	Nostoc linckia AICB 421
chlorophyll a	4.84
carotenoids	0.843
chlorophyll / carotenoids	5.74
Phycobiliproteins:	
phycocyanin	2,464
allophycocyanin	3,385
C-phycoerytrin	3,352

The chlorophyll fluorescence is emitted mostly by PS II and reflects the processes that take place in the light harvesting antenna and in the reaction center. The initial fluorescence F_0 is provided only by the antenna, while the transfer from F_0 to F_m is due to the reaction center (Lichtenthaler and Babani, 2004). In dark adapted probes, the reaction centers are "opened", and the primary acceptors Q_A and Q_B are in oxidized state. After the exposure to a high irradiance, the energy of the absorbed photons is

transferred through the excitone way from the antenna to the reaction center, where the charges are separated, Q_A and Q_B being consequently reduced, thus the reaction center becomes "closed". If no prolonged charge separation occurs, the absorbed energy is emitted as fluorescence, thus F_m is reached. The PS II photosystem is involved in the photosynthetic conversion of the photons which is measured as qP photochemical coefficient (Lichtenthaler *et al*., 2005).

Under the effect of 800 μmol.m^{-2}.s^{-1} light intensity in *Nostoc linckia* AICB 421, the minimal fluorescence F_0 had an oscillating evolution relative to control (fig. 2). The basal fluorescence shows that the primary acceptor Q_A is in oxidized state and the reaction centers are opened. The photochemical process and the harvest of light are maximal. F_m decreased gradually, with the exception of a 107.8% rising after 30 min of light exposure. Due to the rising of the fluorescence from the basal level F_0 to the maximal value F_m, the primary acceptor (Q_A) becomes entirely reduced and the photochemistry is blocked, the reaction centers becoming closed. At the end of the light exposure (two hours) the decreasing of the maximal fluorescence was about 93%. The changes in F_0 and F_m determined the lowering of the variable fluorescence Fv.

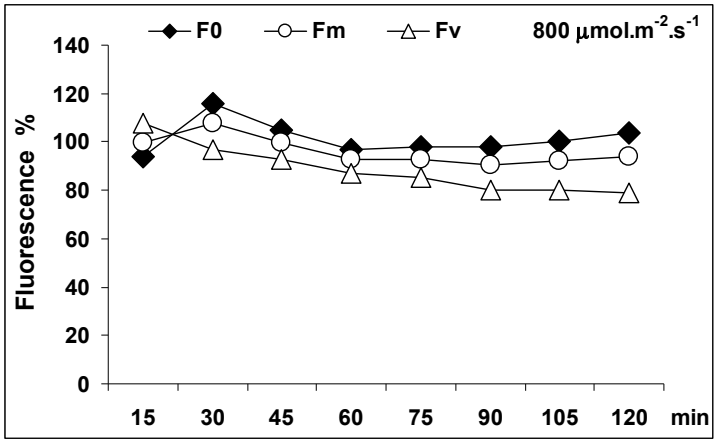

Figure 2. The effect of 800 μmol.m^{-2}.s^{-1} light intensity on the evolution of chlorophyll fluorescence parameters at *Nostoc linckia* AICB 421.

The maximal quantum yield (F_v/F_m) of PS II photosystem, gradually decreased, with the exception of the first 15 min. of light exposure (fig. 3). The reduction of the fluorescence in the end was of 84.4% relative to control. F_v/F_m has a theoretical value of 0.82 and indicates the photons maximum fraction which was absorbed in photochemistry. The values below 0.8 indicate the proportion of the reaction centers which are photoinhibited.

The effective quantum yield of PS II, Y(II), decreased to 85% relative to control at the end of the light exposure, with the exception of the first 15 min. The low values assert the converting of the absorbed light energy as chemical energy through the separation of the photochemical charges in the reaction centers. The quantum yield on non-regulated energy dissipation Y(NO), increased to 111% relative to control at the end of the exposure. In the first 15 min. of light exposure, the light dissipation decreased below the control values. The large value of Y(NO) indicates that both the photochemical energy conversion and the protective regulating mechanisms are inefficient.

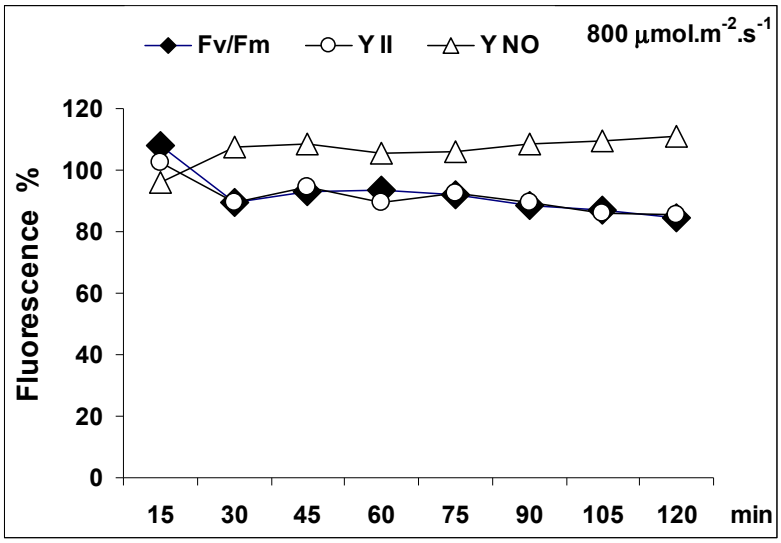

Figure 3. The effect of 800 µmol.m^{-2}.s^{-1} light intensity on the evolution of quantum yields at *Nostoc linckia* AICB 421**.**

The photochemical coefficients qP and qL, maintained a maximum value, with the exception of slightly diminished values at 15 and 60 min. from the light exposure (fig. 4). The qP coefficient is the measure of all openings and the recorded high values assert the opened state of the reaction centers. The photochemical coefficient qL shows the ratio of the opened reaction centers and the obtained high values reflect the high ratio of them. The qP coefficient allows the estimation of the fraction of the oxidized quinone acceptor in PS II or the opened PSII reaction centers (Grace and Logan, 1991).

The high ratio of the opened reaction centers shows that the photochemical activity takes place at high intensities, but the photoinhibition from the antenna determine the lowering of the absorbed excitation energy.

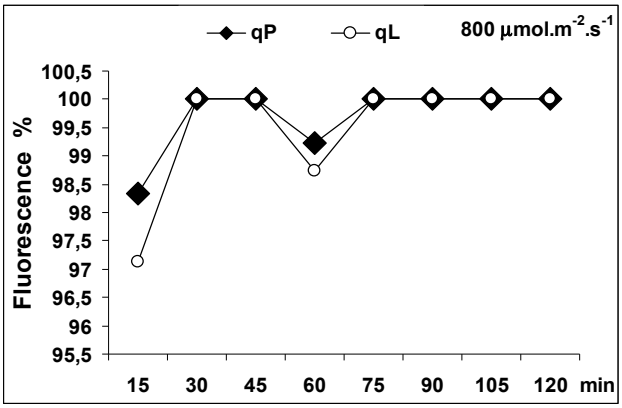

Figure 4. The effect of 800 µmol.m^{-2}.s^{-1} light intensity on the evolution of photochemical coefficients at *Nostoc linckia* AICB 421.

The effect of 1500 µmol.m^{-2}.s^{-1} on the evolution of the chlorophyll fluorescence parameters is shown in fig. 5. F_0 decreased relative to control, with the exception of the high values at 60, 90 and 120 min. F_m decreased proportionally with the duration of exposure, reaching a final decreasing of 89% relative to control. The difference between F_m and F_0 has led to low values for F_v (fig. 5,A)

The maximal quantum yield F_v/F_m of PS II photosystem decreased to a final declining of 63% relative to control (fig. 5,B). The quantum yield on non-regulated energy dissipation, Y(NO), increased reaching a final increase of 125, 6% relative to control. The effective quantum yield of PS II photosystem, Y(II) decreased proportionally with the duration of the exposure, reaching a finale declining of 64.9 % relative to control. The decay of the fluorescence parameters and the quantum yield asserts the inhibition at the antenna and at the PS II reaction centers.

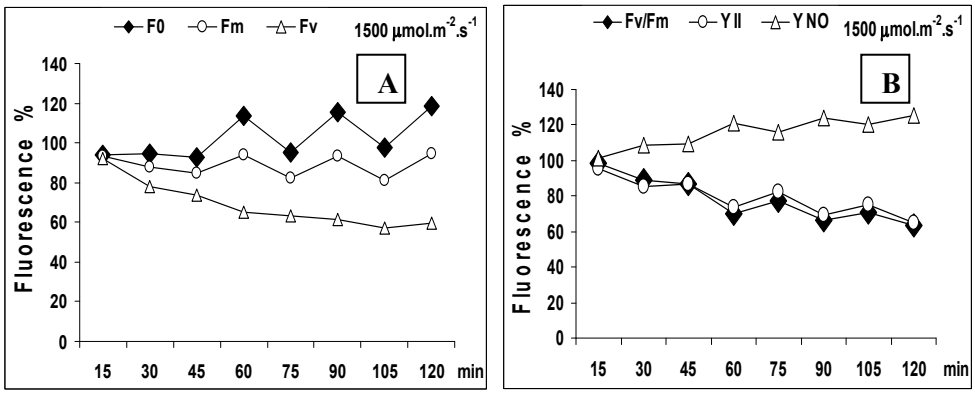

Figure 5. The effect of 1500 µmol.m^{-2}.s^{-1} light intensity on the evolution of the chlorophyll fluorescence (A) and quantum yields (B) at *Nostoc linckia* AICB 421.

The coefficients of photochemical quenching, qP and qL, reached slightly lower values in the first 30 min. of light exposure, than increased to maximum values which remained constant (fig. 6). These high values assert the opened state of the reaction centers and the oxidized state of the primary acceptors of PS II respectively.

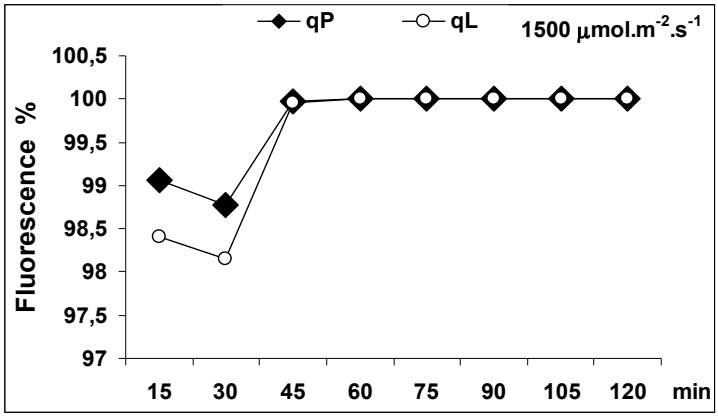

Figure 6. The effect of 1500 µmol.m^{-2}.s^{-1} light intensity on the evolution of the photochemical coefficinets at *Nostoc linckia* AICB 421.

Exposure to a light of 2100 µmol.m^{-2}.s^{-1} produced the increasing of the minimum fluorescence, except for final declining of 97.6%, relative to control (fig. 7). The maximal fluorescence increased above the control values, except the declines recorded in the first 15 min and at the end of the exposure when a diminishing of 84.5% was observed. The differences between the values of these parameters produced the decreasing of the variable fluorescence.

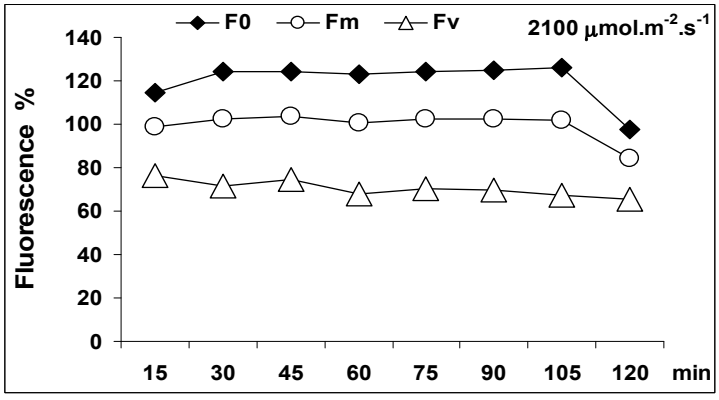

Figure 7. The effect of 2100 µmol.m^{-2}.s^{-1} light intensity on the evolution of the chlorophyll fluorescence parameters at *Nostoc linckia* AICB 421.

The maximal quantum yield F_v/F_m of PS II photosystem, decreased with the exposure duration, reaching a maximal declining of of 66 % relative to control (fig. 8). The effective quantum yield Y(II) of PS II photosystem significantly lowered during light exposure, reaching a declining of 68% relative to control. The quantum yield on non-regulated energy dissipation Y(NO) increased with the exposure duration, reaching an increasing of 123.4% relative to control, after 105 min of light exposure.

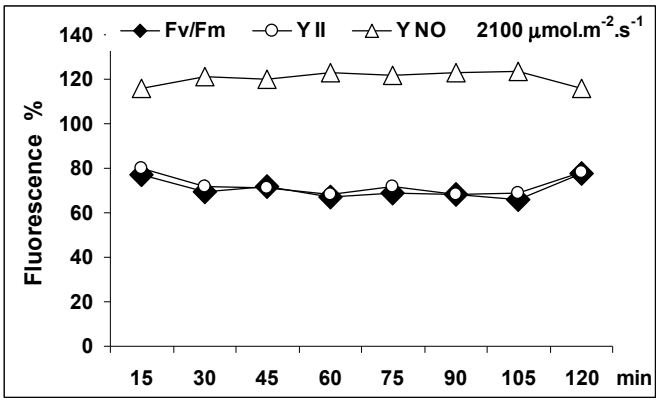

Figure 8. The effect of 2100 µmol.m^{-2}.s^{-1} light intensity on the evolution of the quantum yields at *Nostoc linckia* AICB 421.

The coefficients of photochemical quenching, qP and qL maintained at maximum value, except for a slightly diminishing at 45 min. of light exposure and at the end of it (fig. 9). qP expresses a high ratio of the opened reaction centers and the rising in the fluorescence level asserts that the photoinhibition occurs at the level of the reaction centers, producing the diminishing of the photochemical activity.

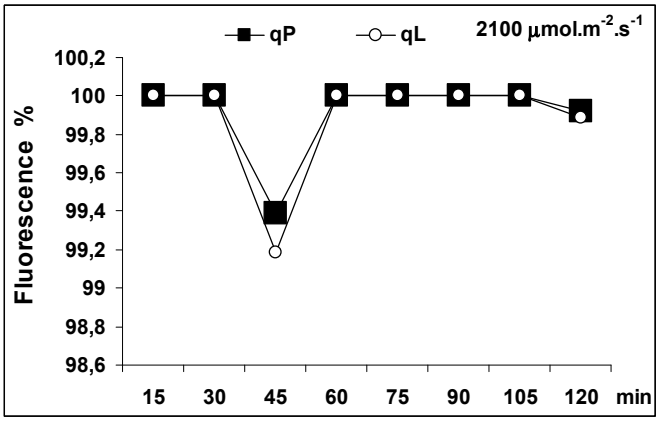

Figure 9. The effect of 2100 µmol.m^{-2}.s^{-1} light intensity on the evolution of the photochemical coefficients at *Nostoc linckia* AICB 421.

The increasing of F_0 certify the lowering of the opened reaction centers, the partial decreasing of plastoquinone in oxidized state and that of F_m, respectively (Joët et. al., 2002). The increasing of F_0, F_m and the quantum yield on nonregulated energy dissipation and the lowering of the quantum yield F_v/F_m, and that of the effective quantum yield Y(II) leaded to photoinhibition induced by 2100 μmoli.m^{-2}.s^{-1} light intensity on the activity developed in the antenna and the reaction centers of PS II.

The cell culture of *Nostoc linckia* AICB 421 which were exposed at different light intensities, were kept in the dark for 120 min, at room temperature in order to study the recovery of the photochemical activity of the PS II photosystem. The minimal fluorescence, F_0, was similar with the control values (fig. 10). The maximal fluorescence decreased, more significantly after the exposure to high intensities of light.

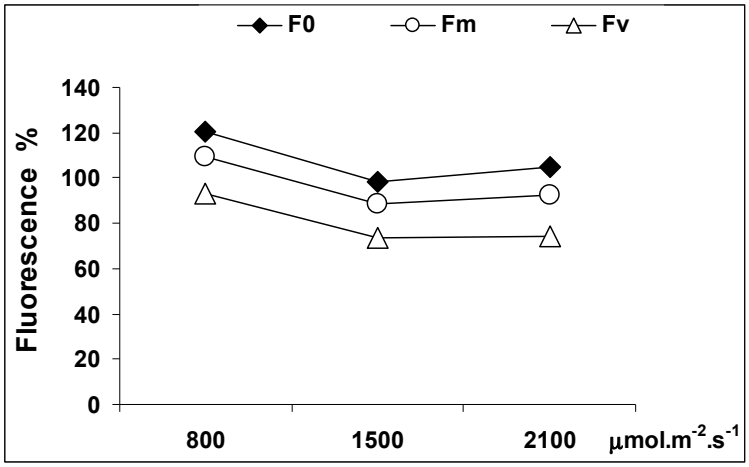

Figure 10. The evolution of the chlorophyll fluorescence parameters at *Nostoc linckia* AICB 421, in recovery phase, after the exposure to various light intensities.

The maximal quantum yield of PS II remained high, with an increasing of 116 % after the exposure to 2100 μmol.m^{-2}.s^{-1}, relative to control (fig. 11). The recovery was slow for both the quantum yield and the quantum yield of non-regulated energy dissipation Y(NO). The effective quantum yield of PS II photosystem remained low and after exposure to 2100 μmol.m^{-2}.s^{-1}, a recovery of 77.9 % was obtained.

The coefficients of photochemical quenching, qP and qL remained at maximal values in the recovery period.

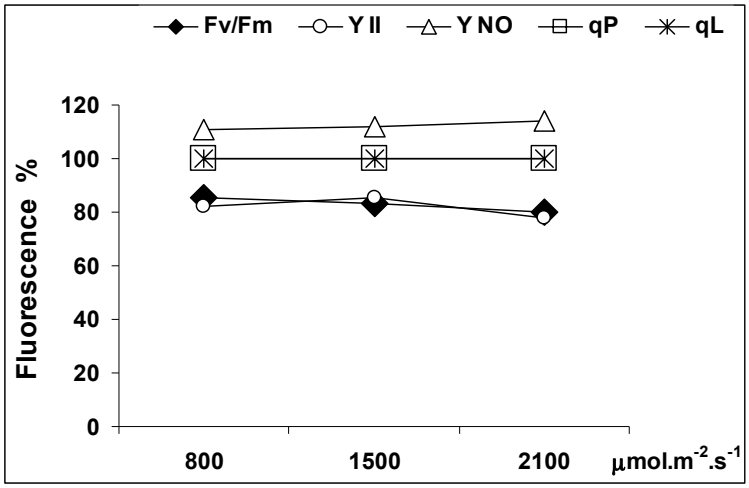

Figure 11. The evolution of the quantum yields and the photochemical coefficients at *Nostoc linckia* AICB 421, in recovery phase, after the exposure to various light intensities.

Conclusions

- *Nostoc linckia* AICB 421 strain was cultivated at room temperature, on GZ medium, under air-lift conditions, using a light intensity of 26 $\mu mol.m^{-2}.s^{-1}$. The cell growth characteristics and the assimilatory pigments content of the photosynthetic apparatus are similar with those found in these type of species. The components of the photosynthetic apparatus are composed of chlorophyll a which together with various types of carotenoids and phycobiliproteins form the light harvesting complex.

- Exposure to 800 $\mu mol.m^{-2}.s^{-1}$ light intensity resulted in registration of oscillating values for F_0 and the declining of F_m producing the diminishing of variable fluorescence. The maximal quantum yield (F_v/F_m) decreased gradually, except for the first 15 min of light exposure and the effective quantum yield Y(II) lowered to 85% in the end. The quantum yield of non-regulated energy dissipation Y(NO) raised significantly. The photochemical coefficients reached maximal values, certifying the opened state of the reaction centers as a result of light exposure. The high ratio of the opened reaction centers shows that the photochemical activity is working in high intensity of light, but the photoinhibitions in the antenna determined the decreasing of the absorbed excitation energy.

- Generally, exposure to 1500 $\mu mol.m^{-2}.s^{-1}$ light intensity, determined the decreasing of F_0 and F_m resulting in low values for F_v. The maximal quantum yield (F_v/F_m) and the effective quantum yield Y(II) lowered with the exposure duration. The quantum yield of non-regulated energy dissipation Y(NO) raised significantly. The photochemical coefficients reached slightly lower values in the first 30 min of

light exposure and subsequently, they reached constant maximum values, certifying the opened state of the reaction centers and the oxidized state of the PS II primary acceptors, respectively.

- Exposure to 2100 $\mu mol.m^{-2}.s^{-1}$ light intensity resulted in the increase of F_0 and F_m, but the variable fluorescence decreased. The maximal quantum yield (F_v/F_m) and the effective quantum yield Y(II) decreased significantly during the exposure. The quantum yield of non-regulated energy dissipation Y(NO) enhanced. The photochemical coefficients maintained at maximum values. qP expresses the high ratio of the opened reaction centers and the increasing level of fluorescence shows that photoinhibition occurs in the reaction centers, determining the diminishing of the photochemical activity.

- The changes in evolution of chlorophyll fluorescence parameters, obtaining low values relative to control, certify the inhibition installation in both the antenna and the PS II reaction centers.

- Recovery of the PS II photosystem photochemical activity was a slow process, depending on the light intensity to which the cell suspensions were previously exposed.

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NEW PCR PRIMERS FOR PARTIAL *YCF*1 AMPLIFICATION IN *ASTRAGALUS* (FABACEAE): PROMISING SOURCE FOR GENUS-WIDE PHYLOGENIES

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SUMMARY: New primer combinations are proposed for PCR amplification and sequencing of an approx. 1.5 kb long fragment from the 3'-end of *Astragalus* hypothetical chloroplast open reading frame 1 (*ycf*1). The efficiency of primers has been tested and confirmed in a wide variety of Old World taxa (including annuals, perennials with basifixed and medifixed hairs) and *Oxytropis pilosa*. The sequenced region showed elevated sequence variability and a consistent phylogenetic potential. *Ycf*1 has been traditionally treated cautiously because of the putative selective pressure acting on it but its popularity as a phylogenetic marker has started to increase recently (applied within angiosperms only in Orchideaceae and Lamiaceae so far). We, therefore, tested for the phylogenetic utility of *ycf*1 in *Astragalus* employing Maximum Parsimony analysis of the 19 *Astragalus* and one *Oxytropis* sequences generated in this study. The resulted tree topology reflected several previously revealed relationships based on ITS sequences of the nuclear ribosomal DNA region or affinities expected from morphology based taxonomic treatments. Taxa of sections *Caprini*, *Dissitiflori* and *Incani* formed monophylous entities, whereas the position of taxa belonging to sections *Craccina* and *Hypoglottidei* revealed the paraphyletic status of these sections. The resulted close relationship between *A. cicer* (sect. *Hypoglottidei*) and *A. onobrychis* (sect. *Onobrychoidei*) warrants further investigations on the re-circumscription of these sections. We propose using *ycf*1 as a phylogenetic marker in *Astragalus* that – especially in combination with other chloroplast markers – might be a useful tool for monophyly testing and sectional delimitation.

Keywords: *Astragalus*, *ycf*1, chloroplast DNA phylogeny

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Introduction

Astragalus L. is the most species rich plant genus among angiosperms with approximately 2,500 species (Lock and Schrire, 2005). Species of this genus are present in all continents, excluding Australia, and have their primary distribution in the northern hemisphere and South America with major centres of diversification in southwest and Central Asia, the Sino-Himalayan region, western North America and in the Andes in South America (Lock and Schrire, 2005). *Astragalus* species are classified in more than 240 sections worldwide: more than 150 in the Old World (Podlech, 1986) and 93 in North America (Barneby, 1964). Sectional classification for the South American species hasn't been established yet (Johnston, 1947). The already defined sections are encompassed within several subgenera whose number depends on different taxonomic concepts (Kazempour Osaloo *et al.*, 2003). Intrasectional relationships in *Astragalus* and those of low-taxonomic levels in general are poorly known. Furthermore, there is an urgent need for revisions of several sections based on molecular phylogenetic approaches in order to build up a modern and in-depth classification of the genus. Previous *Astragalus* molecular phylogenetic studies of the last two decades were aimed at the circumscription of the whole genus, including the pioneer works which reconstructed main extrageneric affinities and intersectional relationships within it (Wojciechowski *et al.*, 1993; Wojciechowski *et al.*, 1999; Kazempour Osaloo *et al.*, 2003; Kazempour Osaloo *et al.*, 2005; Wojciechowski, 2005; Kazemi *et al.*, 2009; Zhang *et al.*, 2009). Nonetheless, after these earlier studies we are now poised to start to understand shallower levels of phylogenetic relationships, as exemplified by the study of Riahi *et al.* (2011), whose goal was to reconstruct the phylogeny of *Astragalus* section *Caprini,* breaking new grounds in *Astragalus* in this respect.

Chloroplast DNA is being successfully used for inferring phylogenies of many plant groups both at high and low taxonomic levels (e.g., Stefanović *et al.*, 2009; Huang and Friar, 2011; Ikinci *et al.*, 2011). The main advantages of chloroplast (cp) markers in phylogenetic inference are: general lack of recombination, single-copy nature, and accessibility to universal primers (Xu *et al.*, 2012). Since plastid DNA is inherited uniparentally, its use (e.g., for sectional delimitation and testing of monophyly) is appropriate because of the lack of biased evolution due to reticulation, albeit in *Astragalus* hybridisation is thought to be rare or non-existing (Liston, 1992; Judd *et al.*, 2008; Kazemi *et al.*, 2009). Chloroplast DNA phylogenies, however, can result in polytomies from analyses of datasets that often lack sufficient levels of sequence variation. Such cases can be explained by the recent and/or rapid diversification of the plant group in question or the use of inappropriate (i.e. relatively slowly evolving) markers.

There is increasing interest in the use of the chloroplast putative open reading frame *ycf*1 as a phylogenetic marker in plant molecular systematics. *Ycf*1 has been repeatedly used in Orchideaceae (Neubig *et al.*, 2008; Chase *et al.*, 2009; Neubig *et al.*, 2012), employed in frame of massively parallel sequencing of chloroplast genomes in Pinaceae (Parks *et al.*, 2009), and employed recently for the first time in the eudicot family Lamiaceae (Drew and Sytsma, 2011). Neubig *et al.* (2008)

found *ycf*1 more variable than *mat*K in Orchideaceae. Similarly, it was roughly 50% more informative than *trn*L-F in a group of taxa belonging to Lamiaceae (Drew and Sytsma, 2011). Parks *et al.* (2009), however, drew attention to the cautious interpretation of *ycf*1 phylogenies because they found evidence for positive selective pressure acting on *ycf*1 in *Pinus* L. Furthermore, Drew and Sytsma (2011) also heightened that the placement, structure and evolution of *ycf*1 need to be treated cautiously since its position at the intersection of one copy of the inverted repeat and the small single copy region could affect its structure.

The objectives of the present study are to: (1) develop a new primer combination for the PCR amplification of a part of *Astragalus ycf*1 showing elevated sequence variability and, consequently, phylogenetic potential; and (2) test the phylogenetic utility of *ycf*1 in *Astragalus*, a chloroplast region which – apart from being phylogenetically informative – was thought to be under selective pressure in another plant group. Since *Astragalus* belongs to the Inverted Repeat Lacking Clade of Fabaceae (Wojciechowski *et al.*, 1999) we hypothesise that the basis of a putative selective pressure on *ycf*1, arising from the fact that this region is located near the inverted repeat, are lacking in *Astragalus*. Furthermore, we will compare our results on the phylogenetic position of some Old World taxa and/or sections either with earlier published molecular phylogenies or with taxonomic treatments based on morphology.

Materials and Methods

a. Taxon sampling

Our sampling strategy was based on the following considerations: (1) to test whether *ycf*1 is amplifiable with our newly devised PCR primers across a wide range of species belonging to different sections and the major morphological categories (annuals, perennials with basifixed hairs, perennials with medifixed hairs) distinguished by Podlech (2008) within European *Astragalus*; (2) to compare different species from the same sections in order to contribute to the second goal of the study. For the sectional placement of taxa we followed the December 2011 updated version of Podlech's (1987) *Thesaurus Astragalorum*. List of taxa used is presented in Table 1. *Oxytropis pilosa* L. was used as outgroup throughout the study.

b. Plant material, DNA isolation, PCR amplification and sequencing

We used both herbarium and field collected material for the investigations (Table 1). For three species DNA extraction was performed from seeds. Total genomic DNA was extracted using the ZR Plant/Seed DNA Kit (Zymo Research). For PCR amplification of an approx. 1.5 kb region from the 3'-end of the plastid *ycf*1 region new (mainly Fabaceae specific) primers were designed (ycf1F-F: ATC MAT GGA CAA RTT GGT T, ycf1F-R: CAA GCT AAA TCT TCT AAT CG) using as a guide the sequences of *Cicer* L., *Glycine* L., *Medicago* L. and *Phaseolus* L. *ycf*1 retrieved

from GenBank. After the first pilot amplifications and sequencing, a more reliable *Astragalus* specific reverse primer (ycf1A-R: CTA ATC GAT AAT TTG GCC) was designed and used for all subsequent reactions. The PCR reaction mixture contained 0.2 volume 5× Green GoTaq Flexi Buffer (Promega), 0.2 mM each of dNTPs (Promega), 3 mM $MgCl_2$, 0.4 μM of each primer (ycf1F-F and ycf1A-R), and 1.25 U GoTaq DNA Polymerase (Promega). Amplification of *ycf*1 required the following "touchdown" PCR reaction program: initial denaturation step at 94°C for 3 min, followed by 38 cycles of denaturation at 94°C for 30 sec, annealing for 1 min and extension at 72°C for 3 min. The initial annealing temperature of 60°C was reduced to 50°C, decreasing it by 1°C per cycle, starting from cycle 2. A final extension at 72°C for 5 min was inserted before final hold at 18°C. All amplifications were performed using a Gradient Palm-Cycler (Corbett Research). PCR products were directly subjected to column purification using Wizard SV Gel and PCR Clean-Up System (Promega) since amplifications did not produce non-specific products (data not shown). DNA sequencing was performed at commercial service provided by Macrogen Inc. using either the amplification primers or three newly designed internal sequencing primers (IntF1: AAA GGA GCA AAC GAA GAA GC, IntF2: AAA TYC CTT TYT TTC CCT T and IntR: TCG TTG AGG TAG TTA TTT CG). GenBank accession numbers for all *ycf*1 sequences generated in this study are listed in Table 1.

c. Phylogenetic analysis

*Ycf*1 sequences from the same accession (obtained with different amplification and/or internal sequencing primers) were assembled using BioEdit (Hall, 1999). Sequences were aligned manually in MEGA5 (Tamura *et al.*, 2011) by taking into consideration the codon positions. The program DnaSP v.5.10.00 (Librado and Rozas, 2009) was used to assess the extent of sequence variation within the sequenced part of *ycf*1 (based on Watterson's θ).

To test for the phylogenetic signal in *Astragalus ycf*1 we performed phylogenetic tree reconstruction using Maximum Parsimony (MP) analysis as implemented in *PAUP** 4.0b10 (Swofford, 2002). The MP analysis used a heuristic search strategy with 1000 random addition of sequence replicates and tree bisection-reconnection (TBR) branch swapping with MULTREES option in effect, MAXTREES set to 15,000 (without the possibility of prompt and auto-increase) and a limit of ten trees retained for each replicate. Characters were weighted equally and gaps were treated as missing data (thus not included in the analysis). The statistical robustness of tree branches was estimated using non-parametric bootstrapping, and is expressed in bootstrap support (BS) percentages. 1000 pseudo-replicates were performed in *PAUP** with heuristic search, TBR swapping, adding the taxa in a simple way and retaining one tree for each replicate. Bootstrap support values were considered as weak (50–74%), moderate (75–84%) and strongly supported (85–100%) (Chase *et al.*, 2000). The trees were visualised and edited in MrEnt v2.2. (Zuccon and Zuccon, 2006).

Table 1. Information on the taxa investigated and plant material used, and list of GenBank acc. numbers for *ycf*1 sequences

Taxon	Section	Habit	Type of plant material	Year of collection	Sampling locality	Voucher (if applicable) or the collector's name	GenBank acc. numbers
A. alpinus L.	Komaroviella	perennial, basifixed	herbarium	2002	Austria, Vorarlberg	W. Lippert (M)	JQ801551
A. cicer L.	Hypoglottidei	perennial, basifixed	seeds[a]	?	Romania, Cluj-Napoca	G. Groza	JQ801552
A. contortuplicatus L.	Cycloglottis	annual	silica dried	2011	Hungary, Nagykörü	G. Sramkó *et al.* (DE)	JQ801553
A. danicus Retz.	Hypoglottidei	perennial, basifixed	herbarium	2003	Germany, Sachsenanhalt	L. & W. Dietrich (M)	JQ801554
A. dasyanthus Pall.	Incerta sedis	perennial, basifixed	silica dried	2011	Romania, Puini	L. Bartha	JQ801555
A. depressus L.	Tapinodes	perennial, basifixed	herbarium	2003	Italy, Piemonte	W. Dietrich (M)	JQ801556
A. dolichophyllus Pall.	Trachycercis	perennial, medifixed	silica dried	2011	Romania, Jurilovca	L. Bartha *et al.* (CL 662383)	JQ801557
A. exscapus L.	Caprini	perennial, basifixed	silica dried	2011	Hungary, Kishartyán	G. Sramkó	JQ801558
A. frigidus (L.) A.Gray	Cenantrum	perennial, basifixed	herbarium	1999	Germany, Oberbayern	H. Förther (MSB 72189)	JQ801559
A. glycyphyllos L.	Glycyphyllos	perennial, basifixed	seeds[a]	?	Romania, Cluj-Napoca	G. Groza	JQ801560
A. hamosus L.	Bucerates	annual	herbarium	1998	France, Verrières	M. Labbé (MSB 91991)	JQ801561
A. hispanicus Coss. ex Bunge	Dissitiflori	perennial, medifixed	silica dried	2011	Spain, Abanilla	L. Bartha *et al.* (CL 662397)	JQ801562
A. monspessulanus L.	Incani	perennial, medifixed	silica dried	2011	Romania, Murfatlar	L. Bartha	JQ801563
A. nummularius Lam.	Caprini	perennial, basifixed	silica dried	2011	Greece, Crete, Kato Simi	L. Bartha *et al.* (CL 662382)	JQ801564

Table 1 *is continued on next page.*

Table 1 (Continued).

Taxon	Section	Habit	Type of plant material	Year of collection	Sampling locality	Voucher (if applicable) or the collector's name	GenBank acc. numbers
A. onobrychis L.	Onobrychoidei	perennial, medifixed	silica dried	2010	Romania, Palazu Mic	L. Bartha	JQ801565
A. scopaeformis Ledeb.	Craccina	perennial, medifixed	silica dried	2009	Ukraine, Novoozerne	L. Bartha	JQ801566
A. spruneri Boiss.	Incani	perennial, medifixed	seeds[a]	?	Romania, Hagieni	G. Groza	JQ801567
A. sulcatus L.	Craccina	perennial, medifixed	herbarium	1985	Austria, Podersdorf	O. Angerer (M)	JQ801568
A. vesicarius L.	Dissitiflori	perennial, medifixed	silica dried	2011	France, Briançon	R. Douzet	JQ801569
Oxytropis pilosa (L.) DC.	-	-	silica dried	2010	Romania, Tănacu	L. Bartha *et al.* (CL 662398)	JQ801550

[a] Seeds obtained from the seed collection of the University of Agricultural Science and Veterinary Medicine, Cluj-Napoca (USAMV)

Results and Discussion

Amplification and sequencing of the *ycf*1 region was successful in all taxa investigated. Complete sequences of the entire region, flanked by the amplification primers, was successful in all cases using the internal sequencing primers. The length of this region varied from 1463 bp (*A. danicus*) to 1523 bp (*A. glycyphyllos*). Sequences assembled from fragments obtained by the amplification primers were at most ca. 30 bp shorter at the 5'- and 3'-ends, respectively, except for *A. frigidus*, whose sequence was missing 205 characters at the more conserved 3'-end (due to some sequencing bias).

The matrix of aligned *ycf*1 sequences contained 1745 characters. One 90 bp long indel differing mainly between the ingroup and *Oxytropis* was excluded from further analyses. The included (1655 bp) part for phylogenetic tree reconstruction had 338 variable (20,42%) and 181 (10,93%) parsimony informative sites. The ingroup (i.e., without *Oxytropis*) contained 309 (18,67%) variable sites including 176 (10,63%) parsimony-informative characters (again disregarding indels). Sequence variation was not uniformly distributed along the region sequenced (Fig. 1). Maximal sequence variability was 'located' broadly between nucleotide positions 750-1500 in the alignment. We also identified two 'hotspots' of sequence diversity consisting of presumably homoplasious indels, which, therefore were not included in the analysis. Data matrix is available upon request from the corresponding author.

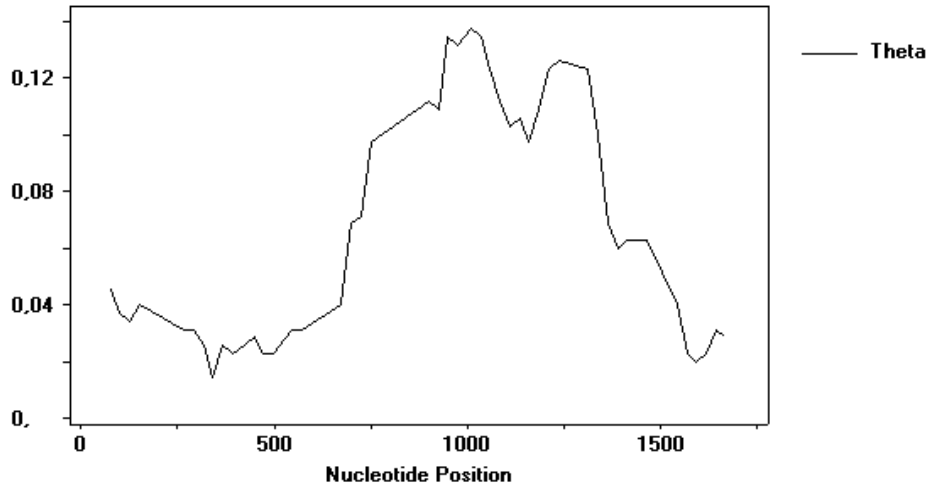

Figure 1. Values of Watterson's theta plotted against alignment position. Estimates were done in 100 bp windows along the aligned *ycf*1 sequences of *Oxytropis pilosa* and 18 *Astragalus* species. *A. frigidus* was omitted because of its 200 bp long missing part of its *ycf*1 sequence.

The heuristic search using the maximum parsimony criterion resulted in six equally most parsimonious trees (tree length=496) of which the strict consensus tree is presented in Fig. 2. The majority of taxa were clustered into three major clades designated by capital letters A-C (Fig. 2). Clade A is the earliest diverging lineage on the tree followed by the polytomy formed by *A. contortuplicatus*, clades B and C. Clades A and C are strongly supported (BS=100% and 99%, respectively) while clade B has only weak (BS=62%) statistical support. Both clades B and C have their well supported subclades (B1, B2 and C1, C2, respectively (Fig. 2)).

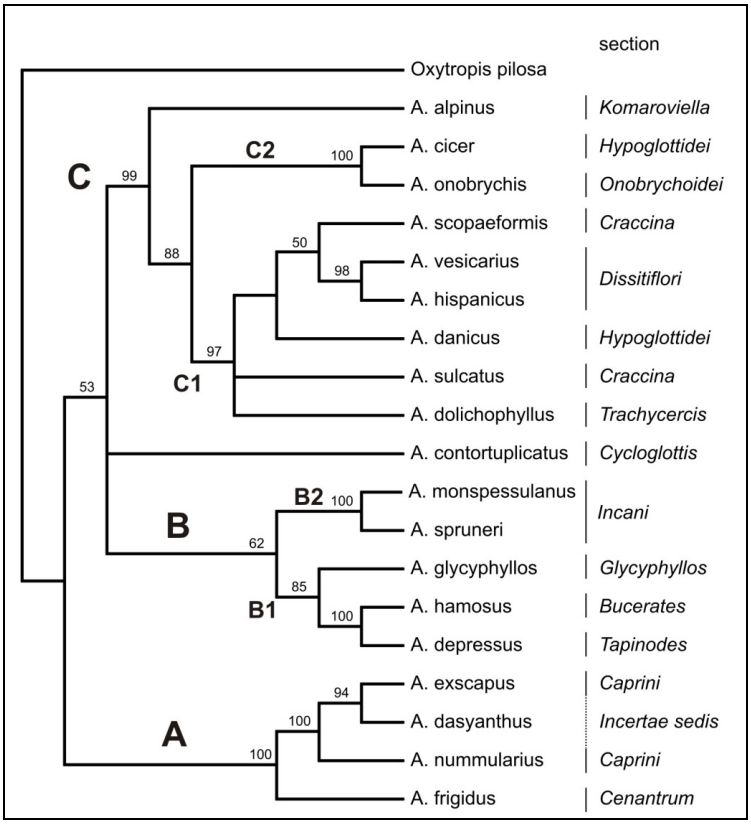

Figure 2. Strict consensus cladogram of the 6 equally most parsimonious trees from the MP analysis 20 *ycf*1 sequences. Numbers above branches represent bootstrap support percentages of 1000 bootstrap replicates.

*Ycf*1 and *Ycf*2 are the largest open reading frames the chloroplast genomes (Drescher *et al.*, 2000) of "higher" plants. The function of these two genes is still unknown (Drew and Sytsma, 2011) but there is evidence they are essential in tobacco where the conceptual translation of these *ORF*s resulted in putative protein products of around 1,901 and 2,280 amino acids, respectively (Drescher *et al.*, 2000).

*Ycf*1 is here for the first time applied as a molecular phylogenetic marker in an analysis of *Astragalus*, the Inverted Repeat Lacking Clade and Fabaceae. Apart from a few exceptions, relationships rendered by parsimony analysis of this region are broadly consistent with previously published molecular phylogenies or with the expected position of some taxa based on classical systematics.

Despite its morphological resemblance to taxa of sect. *Caprini Astragalus dasyanthus* is a species treated as *Incertae sedis* status in Podlech (1987, 2008). Our phylogeny resolved this species with high statistical certainty as sister to *A. exscapus*, and these two species were further resolved (BS=100%) as being sister to *A. nummularius*, also from section *Caprini*. This topology unequivocally supports the proposal to include *A. dasyanthus* in sect. *Caprini*.

In two more cases (sections *Incani* and *Dissitiflori*) taxa from the same sections reflected close relationships, all species of these sections forming well-supported monophyletic groups, therefore corroborating the taxonomic view on their classification. Representative species of section *Craccina*, however, were resolved as polyphyletic, therefore arguing against their current taxonomic treatment. In a previous study of the systematics of Old World *Astragalus* (Kazempour Osaloo *et al.*, 2005) based on nrDNA ITS sequences the phylogenetic position of *A. dolichophyllus* (sect. *Trachycercis*) was unresolved forming a polytomy with species of sect. *Dissitiflori*, sect. *Craccina* and several other sections in a subclade within the so-called clade "F" (sensu Kazempour Osaloo *et al.,* 2003). Our *ycf*1 phylogenetic results corroborate the ITS study in confirming the close relationship between these sections. Nevertheless, the sister relationship of *A. scopaeformis* with the *Dissitiflori* lineage in our phylogeny has only weak (BS=50%) support, and more species would be needed for avoiding the caveat of bias due to insufficient taxon sampling. The two species of section *Hypoglottidei* also have disparate placements in our *ycf*1 phylogeny. *A. danicus* is nested within the well supported (BS=97%) clade C1. On the other hand, *A. cicer* is resolved as sister to *A. onobrichis* (sect. *Onobrichoidei*) with maximal statistical support (clade C2, BS=100%). Such a pattern can only be explained by the paraphyly of section *Hypoglottidei* as currently recognised; another finding that echoes the finding of Kazempour Osaloo *et al.* (2003). Interestingly, Wojciechowski *et al.* (1999) also found *A. cicer* as sister to another species of sect. *Onobrichoidei* (*A. chaborasicus* Boiss. & Hausskn., syn. *A. aduncus* Willd. (Podlech, 1987)). Furthermore, all sections of which taxa are nested within clades C1 and C2 in our phylogeny, are grouped together in the clade "F" sensu Kazempour Osaloo *et al.* (2003), which strongly suggests the same signal in ITS and *ycf*1 for this phylogenetic relationship.

A. glycyphyllos is the first branching lineage in the well supported clade "C" of Kazempour Osaloo *et al.* (2003) of selected Old World *Astragalus* based on ITS sequences of nrDNA. This taxon is followed by the perennial *A. depressus* (as part of a polytomy) and annual *A. hamosus* (occupying one of the terminal positions)

in that clade (Kazempour Osaloo *et al.*, 2005). Our *ycf*1 phylogeny also resolved *A. depressus* and *A. hamosus* as being closely related and *A. glycyphyllos* as an earlier branching taxon sister to the *hamosus-depressus* lineage (clade B1, BS=85%).

Representatives of sect. *Incani* in the phylogeny of Kazempour Osaloo *et al.* (2005) have a very distinct position (in clade "H") and were resolved as sister to sect. *Laguropsis* with high confidence. Because of the lack of species of sect. *Laguropsis* in our phylogeny, the uncertain position of *Incani* species (sister to clade B1 with low statistical support, BS=62%) was to be expected. Compared to our results, clade "H" of Kazempour Osaloo *et al.* (2005) is branching, however, between clades "E" (containing *A. alpinus*) and "F". Thus, the expected position of sect. *Incani* would have been between *A. alpinus* and subclades C1 and C2 in our phylogeny. Our dataset, however, is hardly comparable with that of Kazempour Osaloo *et al.* (2003, 2005) with regard to the extent of taxon sampling, a fact that can serve as a basis for such discrepancies between their and our phylogenies. On the other hand, their clade "D" (containing *A. alpinus*, the well supported *Incani-Laguropsis* lineage and clade "F") is only weakly (BS=55%) supported in Kazempour Osaloo *et al.* (2005) whereas clade C in our phylogeny (containing *A. alpinus*, the "clade F sections", and excluding section *Incani*) is highly (BS=99%) supported, which warrants further investigations on the infrageneric position of sect. *Incani*.

Astragalus contortuplicatus, an annual species belonging to the monotypic section *Cycloglottis,* was not resolved as being closely related to either of the subclades B or C revealed in our study (as being part of a weakly (BS=53%) supported basal polytomy). Our purpose for including this species was to test the PCR amplification of *ycf*1 also in this monotypic section. Regardless, the paraphyletic position of the two annual species (*A. contortuplicatus* and *A. hamosus*) on our *ycf*1 tree also supports the finding of Kazempour Osaloo *et al.* (2003) who concluded the independent evolutionary origin of annual life-form in *Astragalus* and, together with the indumentum type (medifixed vs. basifixed), does not support the subgeneric delimitation of Podlech (1994).

Clade "A" sensu Kazempour Osaloo *et al.* (2003, 2005) is the earliest diverging lineage within *Astragalus* s. str. (Wojciechowski *et al.*, 1999) and comprises species from 13 sections in their phylogenies out of which sect. *Cenantrum* (including *A. frigidus*) is branching second followed by other sections including sect. *Caprini* in their tree. We included only taxa from sections *Caprini* and *Cenantrum* out of those sections but the topology formed by these taxa are in full concordance with that of earlier published phylogenies mentioned above.

Conclusions

In light of the recovery of several previously revealed infrageneric relationships in *Astragalus* by our *ycf*1 phylogeny, we propose the use of this region as an informative marker for phylogeny reconstruction in the genus. In addition to showing a reasonable

number (~10%) of parsimony informative sites within the taxa investigated, *ycf1* sequences are easy to align, an advantage highlighted by authors of previous studies. One can compare its variability with other chloroplast markers already used or not in *Astragalus* phylogenetics. For example, the resolving power of *ycf*1 is similar to that of nrDNA ITS, one of the most frequently utilised genetic marker in plant phylogenetics at lower taxonomic levels (Álvarez and Wendel, 2003) due to its exceptionally fast rate of substitutions (Calonje *et al.*, 2008). Therefore, *ycf*1 can serve as an alternative to nrDNA ITS, where serious problems related to its unusual molecular evolution (pseudogenes, etc.) has been reported (Nieto Feliner and Roselló, 2007).

We do not find evidence for unambiguously aberrant relationships that might have resulted from positive selective pressure acting on this region in *Astragalus*. The unexpected placement of *A. cicer* as sister to *A. onobrichis* warrants further investigations and could be the starting point of re-circumscription of both sections *Onobrychoidei* and *Hypoglottidei*. Thus, *ycf*1 appears to be valuable especially for sectional delimitation, however, its utility at even lower taxonomic levels cannot be excluded. Our ongoing investigations on sect. *Dissitiflori* (Bartha *et al.*, unpublished data) will reveal more insights into the utility of this marker at lower taxonomic levels.

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ON THE PERIODICITY OF AN EPIDEMIC SYSTEM WITHOUT IMMUNITY

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SUMMARY: We introduced a model for some diseases which have temporary immunity. It is to be noted that we paid attention to people who are born and die because of any reasons except of the disease too. Now we prove that the disease is periodic.

Keywords: Susceptible and infective people, SIR Model, periodic model.

Introduction

Nowadays studying about health and contagious diseases is one of the most important issues. One of the good ways to get this aim is studying of mathematical modeling for disease. Maybe, these Models help us to find a logical way for advancement in the future. Mathematical modeling is a good instrument for analysis of SIR models. For the first time Kermak and Mackendrich introduced a basic SIR model (Kermack and McKendrick, 1927) to study the law of the black plague. Now you can find the SIR models so developed where there exists some introductory calculus text books about (Hughes-Hallett *et al*., 2002). From the first paper in 1927 (Kermack and McKendrick, 1927) up to now, many diseases were characterized by SIR models where are goods to research (Bailey, 1975; Mena-Lorca and Hethcote, 1992; Mollison, 1985; Roberts and Kao, 2002; Shi *et al.*, 2009). It is to be noted, there may be a latent period of time, for some disease like AIDS. Many mathematical model of this particular disease where presented by Hethcote and Yorke (1984). SIR Models play such important role in characterization of the disease where can applied to consider the effect of social phenomenon like immigration (Mohamadhasani and Haveshki, 2011b).

Also we introduced an SIR Model which can be demonstration for some diseases which have temporary immunity (Mohamadhasani and Haveshki, 2011a). In this model, as a reality, the children who are born are assumed as a part of susceptible group. It is to be noted that there are some people who die but the reasons of death is

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not the disease. Now we consider the model in section 2 and in section 3 we show our model is periodic. The periodic model is a good instrument to program in the society. Absolutely, it can be helpful for preventing from the disease.

The SIR Model Without Immunity

In this model (Mohamadhasani and Haveshki, 2011a) the population is divided into some groups. The first group is susceptible people. Denoting them by $S(t)$ at time t. We have one assumption. Every member of susceptible people has the same chance to get disease. It means the disease is well-stirred, meaning that every individual has an equal chance to meet the other member of the population. The second group is infective people which is denoted by $I(t)$ at time$t$. Also, there is another group which is called immune people. It is denoted by $R(t)$ at time t. The immune people are not immune for all the time, because the disease dose not have permanent immunity. $D(t)$ is denoted for people who die because of the disease at time t. We consider there are some people who die because of any reasons except of the disease, let $f_1(t), f_2(t)$ and $f_3(t)$ be the number of susceptible people, infective people and immune people who die because of any reasons except of the disease, respectively at time t. There is a positive quantity which called infection rate $r > 0$. There is another positive quantity which called recovery rate $b > 0$. c is another quantity which called dead rate because of disease. Finally $a > 0$ is the last positive quantity which called remove rate. This quantity distinguishes the rate of entering of immune people into susceptible group. $g(t)$ is denoted for the number of people who are born at time t. We assume the people who are born, are just, susceptible people. Finally the differential equations system for the disease is

$$\dot{S} = -rSI + aR - \dot{f}_1 + \dot{g}$$

$$\dot{I} = rSI - bI - cI - \dot{f}_2$$

$$\dot{R} = bI - \dot{f}_3 - aR$$

$$\dot{D} = cI$$

The initial conditions attached to the system is $S_0 = S(0) > 0, I_0 = I(0) > 0$ and $R_0 = R(0) > 0.$

The Periodicity of the Proposed Model

Theorem. The Model has a periodic time.
Proof. By fourier series (Riley *et al.*, 2003), if

$$S(t) = \frac{a_0}{2} + \sum_{n=1}^{\infty} a_n \cos(n\omega t) + \sum_{n=1}^{\infty} a_n' \sin(n\omega t)$$

$$I(t) = \frac{b_0}{2} + \sum_{n=1}^{\infty} b_n \cos(n\omega t) + \sum_{n=1}^{\infty} b_n' \sin(n\omega t)$$

$$R(t) = \frac{c_0}{2} + \sum_{n=1}^{\infty} c_n \cos(n\omega t) + \sum_{n=1}^{\infty} c_n' \sin(n\omega t)$$

and

$$D(t) = \frac{d_0}{2} + \sum_{n=1}^{\infty} d_n \cos(n\omega t) + \sum_{n=1}^{\infty} d_n' \sin(n\omega t)$$

for some $a_0, a_n, a_n', b_0, b_n, b_n', c_0, c_n, c_n', d_0, d_n, d_n' \in R$, for all $n \in N$ and $\omega = \frac{2\pi}{T}$, for all $t \in R^+$, then $S(t), I(t), R(t), D(t)$ are periodic with periodic time $T > 0$. We check if there are such coefficients $a_0, a_n, a_n', b_0, b_n, b_n', c_0, c_n, c_n', d_0, d_n, d_n' \in R$, such that

$$S(t) = \frac{a_0}{2} + \sum_{n=1}^{\infty} a_n \cos(n\omega t) + \sum_{n=1}^{\infty} a_n' \sin(n\omega t) \qquad (1)$$

$$I(t) = \frac{b_0}{2} + \sum_{n=1}^{\infty} b_n \cos(n\omega t) + \sum_{n=1}^{\infty} b_n' \sin(n\omega t) \qquad (2)$$

$$R(t) = \frac{c_0}{2} + \sum_{n=1}^{\infty} c_n \cos(n\omega t) + \sum_{n=1}^{\infty} c_n' \sin(n\omega t) \qquad (3)$$

$$D(t) = \frac{d_0}{2} + \sum_{n=1}^{\infty} d_n \cos(n\omega t) + \sum_{n=1}^{\infty} d_n' \sin(n\omega t) \qquad (4)$$

and also

$$\dot{S} = -rSI + aR - \dot{f}_1 + \dot{g} \tag{5}$$

$$\dot{I} = rSI - bI - cI - \dot{f}_2 \tag{6}$$

$$\dot{R} = bI - \dot{f}_3 - aR \tag{7}$$

$$\dot{D} = cI \tag{8}$$

or not.
By (1), (2), (3), (4) we have

$$\dot{S}(t) = -\sum_{n=1}^{\infty} n\omega a_n \sin(n\omega t) + \sum_{n=1}^{\infty} n\omega a_n' \cos(n\omega t) \tag{9}$$

$$\dot{I}(t) = -\sum_{n=1}^{\infty} n\omega b_n \sin(n\omega t) + \sum_{n=1}^{\infty} n\omega b_n' \cos(n\omega t) \tag{10}$$

$$\dot{R}(t) = -\sum_{n=1}^{\infty} n\omega c_n \sin(n\omega t) + \sum_{n=1}^{\infty} n\omega c_n' \cos(n\omega t) \tag{11}$$

$$\dot{D}(t) = -\sum_{n=1}^{\infty} n\omega d_n \sin(n\omega t) + \sum_{n=1}^{\infty} n\omega d_n' \cos(n\omega t) \tag{12}$$

By (8), (2) and (12) we get

$$-\sum_{n=1}^{\infty} n\omega d_n \sin(n\omega t) + \sum_{n=1}^{\infty} n\omega d_n' \cos(n\omega t) = \frac{cb_0}{2} + \sum_{n=1}^{\infty} cb_n \cos(n\omega t) + \sum_{n=1}^{\infty} cb_n' \sin(n\omega t)$$

as the functions $\cos x$, $\sin x$ and fixed functions are independent, then we get

$$-n\omega d_n = cb_n' \tag{13}$$

$$n\omega d_n' = cb_n \tag{14}$$

$$\frac{cb_0}{2} = 0 \tag{15}$$

By (2), (3), (11) and (7) we have

$$-\sum_{n=1}^{\infty} n\omega c_n \sin(n\omega t)+\sum_{n=1}^{\infty} n\omega c_n{}' \cos(n\omega t)=$$

$$\frac{bb_0}{2}+\sum_{n=1}^{\infty} bb_n \cos(n\omega t)+\sum_{n=1}^{\infty} bb_n{}' \sin(n\omega t)-f_3-\frac{ac_0}{2}-\sum_{n=1}^{\infty} ac_n \cos(n\omega t)-\sum_{n=1}^{\infty} ac_n{}' \sin(n\omega t)$$

By this assumption

$$f_3 = x_0+\sum_{n=1}^{\infty} x_n \cos(n\omega t)+\sum_{n=1}^{\infty} x_n{}' \sin(n\omega t),\ x_0, x_n, x_n{}' \in R \text{ for all } n\in N \text{ . (*)}$$

which meaning f_3 is periodic, we have

$$-c_n n\omega = bb_n{}' - x_n{}' - ac_n{}' \tag{16}$$

$$c_n{}' n\omega = bb_n - x_n - ac_n \tag{17}$$

$$\frac{bb_0}{2}-x_0-\frac{ac_0}{2}=0 \tag{18}$$

by (1), (2), (6) and (10) we get

$$-\sum_{n=1}^{\infty} n\omega b_n \sin(n\omega t)+\sum_{n=1}^{\infty} n\omega b_n{}' \cos(n\omega t)=$$

$$r(\frac{a_0}{2}+\sum_{n=1}^{\infty} a_n \cos(n\omega t)+\sum_{n=1}^{\infty} a_n{}' \sin(n\omega t))(\frac{b_0}{2}+\sum_{n=1}^{\infty} b_n \cos(n\omega t)+\sum_{n=1}^{\infty} b_n{}' \sin(n\omega t))-$$

$$(b+c)(\frac{b_0}{2}+\sum_{n=1}^{\infty} b_n \cos(n\omega t)+\sum_{n=1}^{\infty} b_n{}' \sin(n\omega t))-f_2$$

by two assumptions:

$$f_2 = y_0+\sum_{n=1}^{\infty} y_n \cos(n\omega t)+\sum_{n=1}^{\infty} y_n{}' \sin(n\omega t),\ y_0, y_n, y_n{}' \in R \quad \text{for all } n\in N \text{ . (**)}$$

and

$$a_n = a_n{}' = 0, \textit{ for all } n\in N . \tag{19}$$

We have

$$-b_n n\omega = r\frac{a_0}{2}b_n{}' - (b+c)b_n{}' - y_n{}' \tag{20}$$

$$b_n{}' n\omega = r\frac{a_0}{2}b_n - (b+c)b_n - y_n \tag{21}$$

$$r\frac{a_0}{2}\frac{b_0}{2} - (b+c)\frac{b_0}{2} - y_0 = 0 \tag{22}$$

finally by this assumption :

$-\dot{f}_1 + \dot{g} = z_0 + \sum_{n=1}^{\infty} z_n \cos(n\omega t) + \sum_{n=1}^{\infty} z_n{}' \sin(n\omega t), \quad z_0, z_n, z_n{}' \in R$ for all $n \in N$. $(***)$

and by (1), (2), (3), (5) and (9) we have

$$-\sum_{n=1}^{\infty} n\omega a_n \sin(n\omega t) + \sum_{n=1}^{\infty} n\omega a_n{}' \cos(n\omega t) =$$

$$-r\left(\frac{a_0}{2} + \sum_{n=1}^{\infty} a_n \cos(n\omega t) + \sum_{n=1}^{\infty} a_n{}' \sin(n\omega t)\right)\left(\frac{b_0}{2} + \sum_{n=1}^{\infty} b_n \cos(n\omega t) + \sum_{n=1}^{\infty} b_n{}' \sin(n\omega t)\right) +$$

$$\left(\frac{ac_0}{2} + \sum_{n=1}^{\infty} ac_n \cos(n\omega t) + \sum_{n=1}^{\infty} ac_n{}' \sin(n\omega t)\right) + z_0 + \sum_{n=1}^{\infty} z_n \cos(n\omega t) + \sum_{n=1}^{\infty} z_n{}' \sin(n\omega t)$$

then by (19)

$$-a_n n\omega = -r\frac{a_0}{2}b_n{}' + ac_n{}' + z_n{}' \tag{23}$$

$$a_n{}' n\omega = -r\frac{a_0}{2}b_n + ac_n + z_n \tag{24}$$

$$-r\frac{a_0}{2}\frac{b_0}{2} + \frac{ac_0}{2} + z_0 = 0 \tag{25}$$

Now, just, it is necessary getting the cofficeints

$a_0, a_n, a_n{}', b_0, b_n, b_n{}', c_0, c_n, c_n{}', d_0, d_n, d_n{}' \in R$, for all $n \in N$.

By (19) $a_n = a_n{}' = 0, for\ all\ n \in N$.

By (15) and this assumption:

$$c \neq 0 \tag{****}$$

We have

$$b_0 = 0 \tag{26}$$

By (13) and (14) we have for all $n \in N$:

$$d_n{}' = \frac{cb_n}{n\omega} \tag{27}$$

$$d_n = -\frac{cb_n{}'}{n\omega} \tag{28}$$

By (18) and (26) we have

$$c_0 = \frac{-2x_0}{a} \tag{29}$$

By (25) and (26) we get

$$z_0 = \frac{-c_0 a}{2} \tag{30}$$

which by (29) we have $z_0 = x_0$. By (22) and (26) we get

$$y_0 = 0 \tag{31}$$

By (20) and (21) we have

$$-b_n n\omega - (\frac{ra_0}{2} - b - c)b_n{}' = -y_n{}'$$

$$b_n{}' n\omega - (\frac{ra_0}{2} - b - c)b_n = -y_n$$

then for all $n \in N$:

$$b_n = \frac{(\frac{ra_0}{2} - b - c)y_n + n\omega y_n{}'}{n^2\omega^2 + (\frac{ra_0}{2} - b - c)^2} \tag{32}$$

$$b_n{}' = \frac{(\frac{ra_0}{2} - b - c)y_n{}' - n\omega y_n}{n^2\omega^2 + (\frac{ra_0}{2} - b - c)^2} \tag{33}$$

Then by (23), (24) and (19) we have for all $n \in N$:

$$c_n = \frac{\frac{ra_0}{2}b_n - z_n}{a} = \frac{\frac{ra_0}{2}\frac{(\frac{ra_0}{2} - b - c)y_n + n\omega y_n{}'}{n^2\omega^2 + (\frac{ra_0}{2} - b - c)^2} - z_n}{a} \tag{34}$$

$$c_n' = \frac{\frac{ra_0}{2}b_n' - z_n'}{a} = \frac{\frac{ra_0}{2}\frac{(\frac{ra_0}{2}-b-c)y_n' - n\omega y_n}{n^2\omega^2+(\frac{ra_0}{2}-b-c)^2} - z_n'}{a} \tag{35}$$

By (16)

$$x_n' = bb_n' - ac_n' + c_n n\omega \tag{36}$$

finally by (17)

$$x_n = bb_n - ac_n + c_n' n\omega \tag{37}$$

by (27), (28), (32) and (33) we have

$$d_n' = \frac{c\frac{(\frac{ra_0}{2}-b-c)y_n + n\omega y_n'}{n^2\omega^2+(\frac{ra_0}{2}-b-c)^2}}{n\omega} \tag{38}$$

$$d_n = \frac{c\frac{(\frac{ra_0}{2}-b-c)y_n' - n\omega y_n}{n^2\omega^2+(\frac{ra_0}{2}-b-c)^2}}{n\omega} \tag{39}$$

By the assumptions $(*),(**),(***)$ and $(****)$ and by arbitrary $a_0, d_0, z_n, z_n', y_n, y_n', x_0 \in R$ for all $n \in N$ we can get a_n, a_n' by (19), b_0, b_n, b_n' by (32), (33), (26). d_n, d_n' by (38), (39) and finally c_0, c_n, c_n' by (29), (34), (35) for all $n \in N$. Also y_0 is gotten by (31), z_0 by (30) and x_n, x_n' by (36), (37). Then the SIR Model is periodic.

Conclusion

In this paper by using of an interesting property in Fourier series, we proved the introduced mathematical model for some disease without immunity is periodic. When an epidemic is periodic, we can predict time of entering the epidemic into the society. This can provide a good view for further research in continuation of characterizing the disease's properties.

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ASSESSING COPING AND LOCUS OF CONTROL IN CARDIOVASCULAR PATIENTS

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SUMMARY: Locus of control is a theory in personality psychology referring to the extent to which individuals believe that they control events that affect them. Locus of control can be internal (subjects believes that they control their life) or external (subjects believe that the environment, some higher power or other people control their life). Coping has been defined as constantly changing cognitive and behavioral efforts to manage disease and its effects on the ill person. The purpose of the study was to assess the locus of control and coping strategies in patients with cardiovascular diseases. The study was performed on 70 patients suffering from various cardiovascular diseases. Statistical processing was performed using Microsoft Office Excel and GraphPad. Patients suffering from arterial hypertension exhibited an internal locus of control; patients with arterial hypertension and other cardiovascular diseases (like coronary heart disease) expressed an external locus of control. Patients with external locus of control cope through active coping, while patients with internal locus of control do not use this mechanism.

Keywords: cardiovascular disease, coping, locus of control.

Introduction

Disease is an abstractization in medical practice, the only concrete and palpable reality being the ill person (Cosman, 2010). The condition cannot be reduced to its purely medical side; it entails a certain experience or an attitude of the patient, sufferance not being isolated, but integrated in the human concepts and internal emotions of the ill person (Manea and Manea, 2004; Lupu *et al.*, 2004). The disease is internalized differently, depending on the context of occurrence, time of life, personality traits, socio-cultural environment, culture level and patient

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history. Sometimes, the belief upon the disease is realistic, based on scientific information, but sometimes, this concept is based on prejudice, absurd and superstition (Cosman, 2010).

Locus of control is a theoretical construct designed to assess and explain a person's perceived control over his or her own behavior or life events. Internal locus of control indicates that the person feels in control of events (or disease), whereas external locus of control defines the lack of control concerning the person's life, the events being attributed to others, to luck, fate or a superior power. It has been proved that locus of control mediates the influence of life events on the person's wellbeing and coping capacity (Vraşti, 2001). People exhibiting an internal locus of control during childhood have a lower risk to develop arterial hypertension or obesity as adults. Thus, the patients who express a greater control on their life events appear more protected from some adulthood diseases (Gale, 2008).

Coping has been defined as a series of awareness strategies to adjust or accommodate to a certain situation (or disease), by constantly changing cognitive and behavioral efforts to manage specific internal or external demands, which exceed the resources of the person (Bradu-Iamandescu, 2005).

Two classifications of coping mechanisms have been developed. The first (Folkman and Lazarus) used the criteria of attention focusing and classified coping mechanisms in:

- **Problem-centered coping (vigilant coping)** – implies concentration on the problem, with the purpose of finding a modality of changing it or avoiding it in the future; it is used in potentially reversible situations;
- **Emotions-centered coping (avoidant coping)** – implies the reduction of event associated emotions, even if the situation cannot be changed.

Another broader classification of coping mechanisms is presented by Carver, Scheier and Weintraub (Carver, 1989):

- *Active coping (AC)* is the process of taking active steps to try to remove or circumvent the stressor or to ameliorate its effects;
- *Planning (P)* is thinking about how to cope with a stressor; it involves coming up with action strategies, thinking about what steps to take and how best to handle the problem;
- *Suppression of competing activities (SCA)* means putting other projects aside, trying to avoid becoming distracted by other events, even letting other things slide, if necessary, in order to deal with the stressor;
- *Restraint coping (Re)* is waiting until an appropriate opportunity to act presents itself, holding oneself back, and not acting prematurely;
- *Seeking social support for instrumental reasons (ISS)* is seeking advice, assistance or information; this is problem-focused coping;

- *Seeking social support for emotional reasons (ESS)* is getting moral support, sympathy, or understanding; This is an aspect of emotion-focused coping;
- *Focusing on and venting of emotions (VE)* – the tendency to focus on whatever distress or upset one is experiencing and to ventilate those feelings;
- *Behavioral disengagement (BD)* implies reducing one's effort to deal with the stressor, even giving up the attempt to attain goals with which the stressor is interfering;
- *Mental disengagement (MD)* is a variation on behavioral disengagement, postulated to occur when condition prevent behavioral disengagement; occurs via a wide range of activity that serve to distract the person from thinking about the behavioral dimension or goal with which the stressor is interfering;
- *Positive reinterpretation and growth* (PRG) is a type of emotion-focused coping: coping aimed at managing distress emotions rather than at dealing with a stressor per se;
- *Denial (D)* is a response that sometimes emerges in primary appraisal – the refusal to believe that the stressor exists or of trying to act as though the stressor is not real;
- *Acceptance (A)* is the opposite of denial. Acceptance of a stressor as real occurs in primary appraisal. Acceptance of a current absence of active coping strategies relates to secondary appraisal. One might expect acceptance to be particularly important in circumstances in which the stressor is something that must be accommodated to, as opposed to circumstances in which the stressor can easily be changed;
- *Turning to religion (R)*. One might turn to religion when under stress for widely varying reasons: religion might serve as a source of emotional support, as a vehicle for positive reinterpretation and growth, or as a tactic of active coping with a stressor;
- *Drugs and alcohol use (S)* interact with the patient's perception on the disease; often, anxiolytic mediation is used.

The aim of the study was to assess the locus of control and coping strategies in patients with cardiovascular diseases.

Materials and Methods

The study was performed on 70 cardiovascular patients (60% males, 40% females), from, Cardiology department, County Emergency Hospital *Medicala I*, Cluj-Napoca, Romania. The study was approved by the ethical committee of the clinic, and all patients agreed with the participation in the study. The average age of the patients was 59±8 years.

Two questionnaires were applied to each patient, concerning:

- Locus of control;
- Coping strategies;
- Smoking habits;
- Living and religious status.

Each question was read to the patient, and explained if needed. Also, medical data was collected for each participant from the hospital records, with the consent of the patient's medical practitioner.

Statistical processing and coefficients calculation was performed using the *Chi square test*, using Microsoft Excel and GraphPad.

Results and discussion

Locus of control. External locus of control was predominantly observed among patients with cardiovascular disease (63%), which means that patients attribute the disease to fate, luck, chance or other superior power. Also, internal locus of control is more common among men and less frequent among women (p=0.02), meaning that men consider disease as a consequence to their own behavior.

Patients with arterial hypertension only exhibit an internal locus of control (they attribute the disease to their personal lifestyle), in contrast with patients with arterial hypertension associated with other cardiovascular diseases, coronary heart disease, valvular cardiopathy, chronic arteriopathy of lower limbs and cardiac failure, which express an internal locus of control (they attribute their more serious diseases to others or to a superior force, like Divinity – Fig. 1).

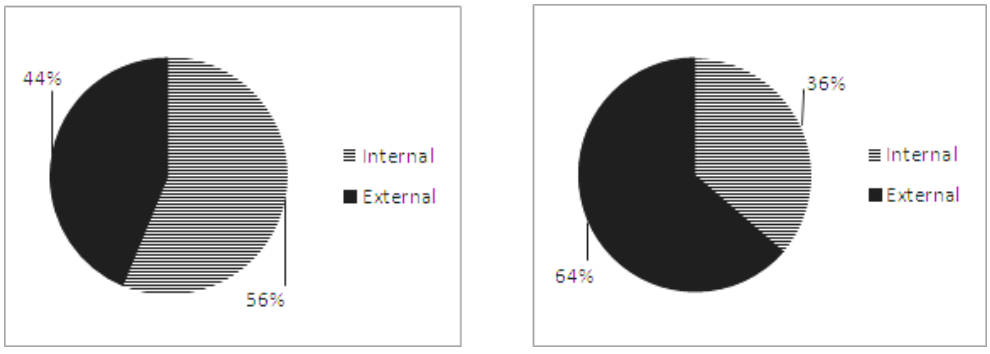

Figure 1. Locus of control of patients with arterial hypertension (left) and with arterial hypertension associated with other diseases (right)

The results are consistent with those of a study conducted in Poland, in 2009. The authors obtained an internal locus of control in patients suffering from arterial hypertension only and an external locus of control in patients suffering from arterial hypertension associated with coronary heart disease (Opuchlik *et al.*, 2009). A possible explanation is that hypertension is a very common disease, with risk factors known to most of the population and it is usually associated with unhealthy lifestyle (internal locus); however, if another cardiovascular disease is associated or other disease is present, patients believe that they have been punished by God, touched by spells or bad luck (external locus of control).

Coping. It has been observed that men use acceptance less frequent than women ($p=0.01$) and women involve in a more active way in fighting with the disease ($p=0.05$). Environment plays an important role in religious coping, as most of the rural patients use religion as a coping mechanism ($p<0.01$). Religious people also tend less to use alcohol and drugs ($p<0.01$) or smoking ($p=0.02$) as coping methods.

Table I expresses the correlations between all types of coping, with $p<0.05$:

- "+" signifies the presence of a concordant association (e.g. patients that cope by instrumental social support also use emotional social support as coping method);
- "-" signifies the presence of a discordant association (e.g. patients coping by positive reinterpretation and growth do not cope using mental disengagement).

Table I – Correlations between different types of coping.

	ISS	***ESS***	***PRG***	***MD***	***BD***	***A***	***D***	***VE***	***R***	***AC***	***P***	***SCA***	***Re***	***S***
ISS		+				+	-	+		+				-
ESS	+					+	-	+		+				
PRG					-				-	+	+		-	
MD							-		+			-		
BD			-							-	-		+	
A	+	+					-							-
D	-	-		-		-								
VE	+	+												
R			-	+							-	-		
AC	+	+	+		-						+		-	
P			+		-				-	+		+	-	
SCA				-					-		+			
Re			-		+					-	-			
S	-					-								

Coping strategies were observed to group in two categories: *adaptive (active)* – instrumental social support, emotional social support, positive reinterpretation and growth, acceptance, emotional discharge, active coping, planning and elimination of competing activities and *non adaptive (passive)* – mental disengagement, behavioral disengagement, denial, religion, restraint and alcohol and drugs abuse.

Patients with cardiovascular diseases use predominantly the following coping strategies: active coping, acceptance, instrumental social support, positive reinterpretation and growth, planning, emotional social support, suppression of competing activities, religion and emotional discharge; they do not use mental passivity, denial, behavioral passivity, restraint and alcohol and drugs abuse (Fig. 2).

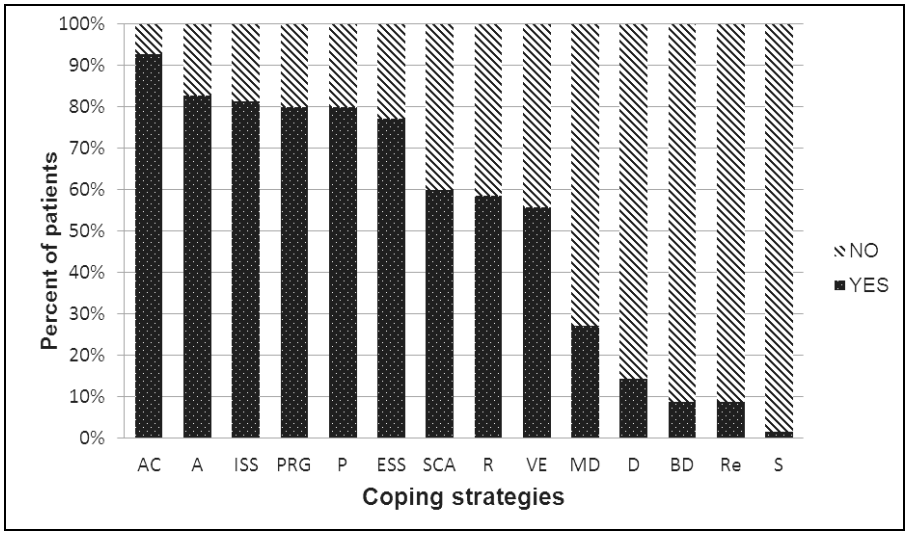

Figure 2. Coping strategies in cardiovascular disease

The results resemble a similar study performed on Romanian students (Băban, 1998). Carvers's results were somewhat different: students use more restraint and less religion (Carver *et al.*, 1989). Differences in religion could be attributed to the fact that the Romanian people are more faithful and often resort to the help of God. Regarding restraint, we might say that Romanians interpret it as a passive coping strategy, therefore they do not use it, while Americans regard it as an active strategy, watchful waiting for the perfect moment to act.

Concerning patients with arterial hypertension and patients with arterial hypertension associated with other cardiovascular diseases, there are differences in coping using emotional discharge and religion (Figures 3 and 4).

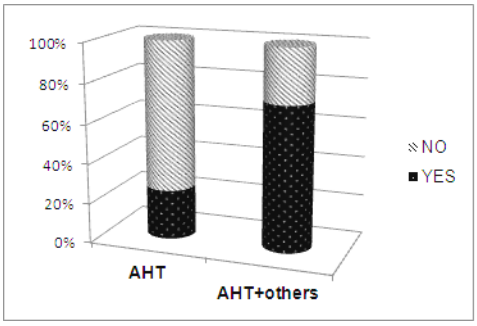

Figure 3. Coping through emotional discharge at patients with AHT and AHT+other diseases (p<0.01)

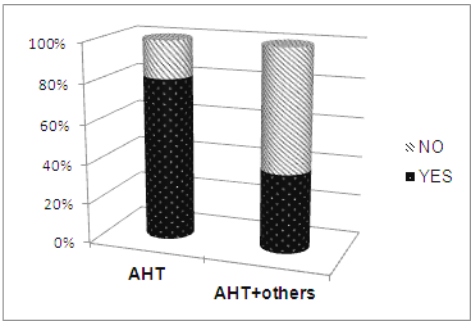

Figure 4. Coping through religion at patients with AHT and AHT+other diseases (p<0.01)

Compared with the general pattern of coping in cardiovascular disease, differences have been observed concerning patients with only one disease. Subjects suffering from arterial hypertension only do not use emotional discharge, possibly because the lack of symptoms, which also determines a very low degree of treatment compliance in these patients.

Patients suffering from arterial hypertension associated with other diseases (coronary heart disease, chronic artheriopaty of the lower limbs and cardiac failure) do not use religion as a coping strategy, possibly because these more serious and symptomatic diseases determine them to loose faith in religion. Patients with a history of myocardial infarction do not cope by suppressing competing activities. We could say that these patients overcame the problem and focus on the future and not on the disease in the past. Valvulopathic patients did not express differences in coping mechanisms compared to the general pattern, possibly because these diseases are less understood by the patients.

Locus of control and coping. It has been observed that patients with external locus of control cope through active coping, while patients with internal locus of control do not use this mechanism (Fig. 5).

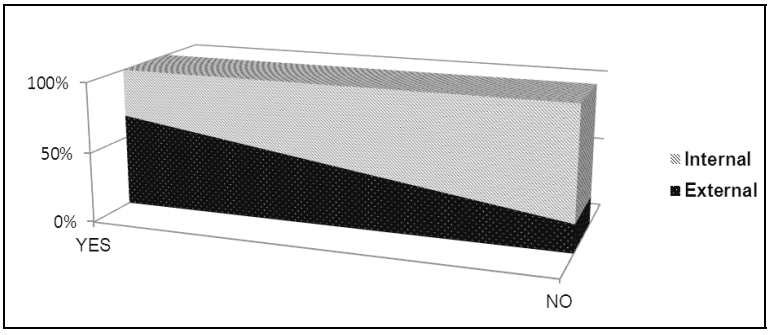

Figure 5. Association betwing locus of control and active coping (p=0,03)

Results resemble those obtained in other studies which described that patients with external locus of control get involved more actively in fighting with the disease (Burish, 1984; Opuchlik *et.al*, 2009; Helgeson, 1992).

Conclusions

1. Locus of control associated with disease coping methods: patients with external locus of control used active coping strategies;

2. Patients suffering from: arterial hypertension associated with other cardiovascular diseases, coronary disease, myocardial infarction, valvular cardiopathy, chronic artheriopathy of the lower limbs and cardiac failure exhibited external locus of control, while patients suffering from arterial hypertension only presented internal locus of control;

3. External locus of control was most prominent among women compared to men;

4. Women tend to use more acceptance and active coping than men;

5. Rural patients, as well as non smoking patients used religion as coping method more than urban and non smoking patients, respectively;

6. Coping methods among patients with cardiovascular diseases were grouped in two categories: *adaptive (active)* – instrumental social support, emotional social support, positive reinterpretation and growth, acceptance, emotional discharge, active coping, planning and elimination of competing activities and *non adaptive (passive)* – mental disengagement, behavioral disengagement, denial, religion, restraint and alcohol and drugs abuse;

7. Cardiovascular patients used the following coping mechanisms: active coping, instrumental social support, positive reinterpretation and growth, planning, emotional social support, elimination of competing activities, religion and emotional discharge and did not use mental disengagement, denial, behavioral disengagement, restraint and alcohol and drugs abuse;

8. Patients suffering from arterial hypertension only did not use emotional discharge, while patients with a history of myocardial infarction did not use elimination of competing activities;

9. Patients with arterial hypertension associated with other cardiovascular diseases, coronary disease, chronic artheriopathy of the lower limbs and cardiac failure did not use religion as a coping strategy.

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LIMNOLOGICAL STUDY OF SEVERAL FRESHWATER LAKES FROM ROŞIA MONTANĂ REGION (TRANSYLVANIA, ROMANIA)

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SUMMARY: The present study includes data on three biotic communities, characteristic to five lakes from the Roşia Montană region, Transylvania, Romania: primary producers (phytoplanktonic algae) and consumers (planktonic crustaceans and benthic invertebrates). The five sampling sites chosen for this paper were located on five man-made shallow lakes: Tăul Anghel, Tăul Brazi, Tăul Cornii, Tăul Mare and Tăul Ţarinii. The samples were collected in 2009 and 2010. The importance of the present study resides in the scarcity of the data from aquatic ecosystems in this particular area, Roşia Montană, whose fate is disputed between mining companies and environmentalists.

Keywords: benthic invertebrates; ecological status; percentage abundance; phytoplankton, planktonic microcrustaceans.

Introduction

The holistic approach of limnological research requires a complete investigation of the communities inhabiting the ecosystem and its ecotonal regions, for a better understanding of the system's functioning. The present paper represents an attempt to follow this approach in the study of several man-made freshwater lakes from Roşia Montană (Transylvania, Romania), because it includes data regarding three major biotic communities. This study deals not only with planktonic communities, both phyto- and zooplankton, which are vital components of lentic food webs, but also with benthic invertebrate communities, that also play an important role, especially in shallow systems.

Roşia Montană area, part of the Alba county, Transylvania, Romania, represents a well-known mining region, whose documented existence dates back to the second century AD. The mining activities carried out here required a constant

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volume of water, in order to handle the equipment for gold ore processing. As a result, about 100 artificial water bodies were created in the 18th century, the largest ones having different mechanisms for water removal and discharge control. These lakes suffered different changes in time: they were emptied and cleaned, or their dams were raised, causing depth increases. However, some of them still exist today, like the five lakes considered for the present study. Even if their initial function was related solely to the mining industry, they represent today a reliable water source during summer time and popular recreational areas for the local community.

There is only one previous study concerning the benthic invertebrate communities from these five lakes: the report provided in 2006 by SC Roşia Montană Gold Corporation S.A. regarding the assessment of human impacts on the environment (”Raport la studiul de evaluare a impactului asupra mediului”). No data were found on planktonic algae or microcrustaceans from the study area.

The aim of the present paper was to investigate the qualitative composition of phytoplankton, planktonic microcrustaceans and benthic invertebrates from five shallow freshwater lakes located in Roşia Montană area.

Material and methods

Five sites were sampled in the Roşia Montană region, one in every freshwater lake considered for the present study. The accurate location of these sampling sites is depicted in Fig. 1, while Table 1 presents the major physical and geographical characteristics of these lakes, including the GPS coordinates, the altitude and basic morphometric data.

Tăul Anghel represents a shallow lake, with a maximum depth of 6 m (Sîntimbrean, 1989), surrounded by trees and shrubs. Tăul Brazi is located near the first lake, at a slightly lower altitude, in the proximity of a touristic site. These two lakes are both surrounded by coniferous and deciduous vegetation. Tăul Cornii is another shallow water body, recording a depth of less than 10 m. In summer, the lake is surrounded by a belt of emerged aquatic macrophytes, while submerged vegetation is well represented too. Tăul Mare is the largest lake considered for the present study, having a maximum depth of 25 m. Tăul Ţarinii is located further north, in an area covered with open forests and meadows; its banks are quite steep and covered with woody and herbaceous vegetation.

The study was conducted in 2009 and 2010. Phytoplankton samples were collected in August 2010 from four lakes (Tăul Anghel, Tăul Brazi, Tăul Cornii and Tăul Ţarinii); while benthic invertebrates were sampled only from two lakes (Tăul Brazi and Tăul Mare), in three seasons: July and November 2009 and April 2010. Data regarding microcrustacean communities included all lakes, as follows: zooplankton samples collected in August 2010 were considered from Tăul Anghel, Tăul Brazi, Tăul Cornii and Tăul Ţarinii, while for Tăul Brazi and Tăul Mare, separate individuals collected in the benthic samples were analyzed.

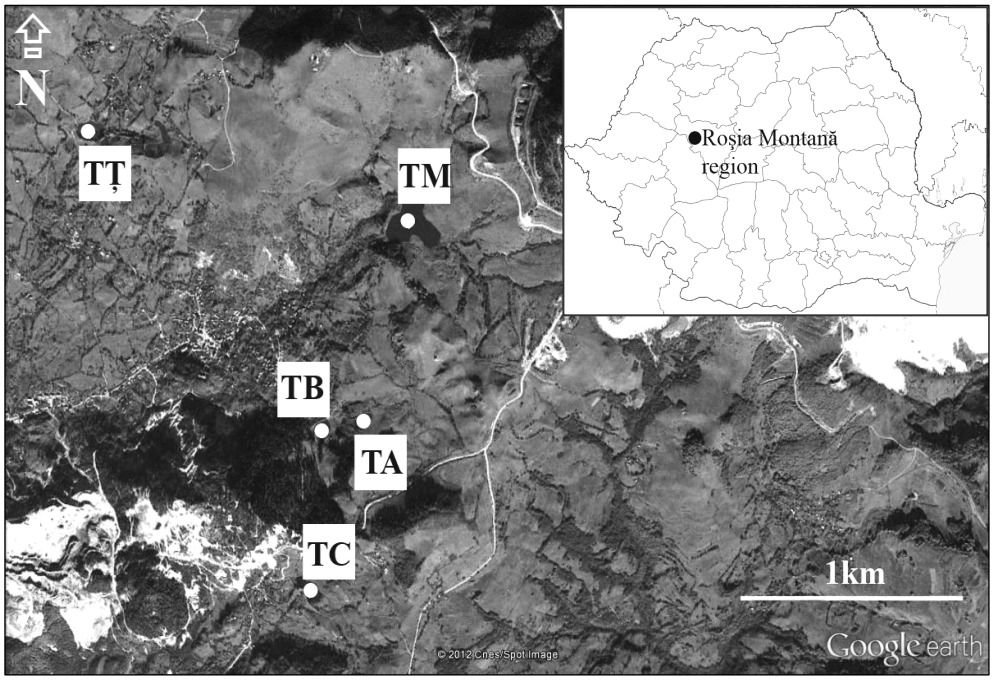

Fig. 1. Location of Roşia Montană region in western Romania (upper right corner) and the position of the five sampling lakes (**TA** - Tăul Anghel; **TB** - Tăul Brazi; **TC** - Tăul Cornii; **TM** - Tăul Mare; **TŢ** - Tăul Ţarinii)

Table 1.

The main characteristics of the five sampling lakes located in Roşia Montană region (after Sîntimbrean, 1989)

Lake	GPS coordinates	Altitude (m)	Total area (m^2)	Total volume (m^3)
TA	N: 46° 18' 10.5" E: 23° 08' 27.1"	992.4	6,000	18,000
TB	N: 46° 18' 07.6" E: 23° 0.8' 16.5"	940.6	9,000	46,000
TC	N: 46° 17' 47.3" E: 23° 08' 14.2"	968	5,000	20,000
TM	N: 46° 18' 38.8" E: 23° 08' 40.0"	1,025	40,000	200,000
TŢ	N: 46° 18' 52.3" E: 23° 07' 25.6"	957.1	5,000	25,000

The most important physical and chemical parameters: the concentration of dissolved oxygen and the water temperature were measured in the field in four sampling sites in August 2010, using a portable meter (YSI 52).

Qualitative samples were collected for both planktonic and benthic communities, by filtering a volume of water from the banks of the water bodies. Because of this sampling method, numerous benthic groups were caught in planktonic samples, especially in case of algal communities. Three types of nets, with different mesh sizes, were used to sample biotic communities: a 40 µm mesh phytoplankton net; a 55 µm mesh zooplankton net and a 250 µm mesh benthic net. The samples were preserved in the field in 4% formaldehyde.

Taxonomical identifications were made to the species level in case of phytoplankton and microcrustacean communities (cladocerans and copepods), while for benthic invertebrates, identifications reached different levels. In case of diatoms, permanent slides were made, following usual methods (Barber and Haworth, 1981).

The relative percentage abundance was calculated for planktonic micro-crustaceans and benthic invertebrates. In case of cladocerans and copepods for example, a certain number of individuals was counted from each sample taken in August 2010, and the percentage of microcrustacean species (copepodites and nauplii included) was estimated using that number as the value of 100%.

For the trophic state assessment, several indices based on phytoplankton were calculated, following the recommendations of Willén (2000): Thunmark index based on Chlorophyta, Nygaard compound index, Heinoen trophic index, and two indices introduced by Oltean (1977): beta- and zeta-eutrophy indices. The last two indices are estimated according to the taxonomical group with algae causing "water blooms". Two organic pollution indices were used to assess the saprobity level in the water bodies: one calculated at the species level (I_S) and the other at the genus level (I_G), according to Willén (2000). Moreover, the saprobic indicator values of certain algal and microcrustacean species were also considered, following Sládeček (1973).

Results and discussion

The physical and chemical parameters measured near the banks in the four lakes in August 2010 (Tăul Anghel, Tăul Brazi, Tăul Cornii, and Tăul Ţarinii) recorded normal values for the sampling period. The water temperature ranged from 22.5°C to 23.7°C, while the dissolved oxygen concentration varied between 6.02 mg/L in Tăul Anghel to 9.14 mg/L in Tăul Cornii, where its saturation reached 108%, probably due to the massive development of algal community that produced high quantity of dissolved oxygen through photosynthesis.

Phytoplankton communities. In Tăul Anghel, Tăul Brazi, Tăul Cornii and Tăul Ţarinii, a total number of 145 algal taxa were identified (Table 2), belonging to 6 phyla: Cyanoprokariota (21 taxa), Euglenophyta (6), Dinophyta (8), Chrysophyta (4),

Table 2.

List of phytoplanktonic species identified in the five lakes from Roşia Montană region (TA - Tăul Anghel; TB - Tăul Brazi; TC - Tăul Cornii; TM - Tăul Mare; TŢ - Tăul Ţarinii; ✓ - present; · - not present)

TAXA	TA	TB	TC	TŢ
Cyanoprokaryota				
Anabaena affinis	·	·	✓	·
Anabaena circinalis	✓	✓	·	·
Anabaena elliptica	·	✓	·	·
Anabaena minima	·	✓	·	·
Anabaena viguieri	·	✓	·	·
Aphanizomenon flos-aquae	·	·	·	✓
Arthrospira jenneri	·	·	·	✓
Gomphosphaeria aponina	✓	·	·	·
Microcystis aeruginosa	✓	·	·	·
Microcystis viridis	·	✓	·	✓
Oscillatoria amphibia	·	·	·	✓
Oscillatoria chlorina	·	·	✓	·
Oscillatoria geminata	·	·	·	✓
Oscillatoria irrigua	·	·	·	✓
Oscillatoria lacustris	✓	·	·	·
Oscillatoria limosa	✓	·	·	·
Oscillatoria planctonica	✓	·	·	·
Oscillatoria pseudogeminata	·	·	·	✓
Oscillatoria putrida	·	✓	·	·
Oscillatoria tenuis	✓	·	✓	·
Phormidium tenue	✓	·	·	·
Euglenophyta				
Euglena texta	·	·	✓	✓
Euglena oxyuris	·	·	✓	·
Phacus longicauda	·	✓	·	·
Phacus tortus	·	✓	·	·
Trachelomonas planctonica	·	✓	·	·
Trachelomonas volvocina	·	✓	·	·
Dinophyta				
Ceratium furcoides	·	✓	✓	·
Ceratium hirudinella	✓	·	·	·
Peridinium aciculiferum	·	✓	✓	✓
Peridinium bipes	·	·	✓	·
Peridinium cinctum	·	✓	✓	·
Peridinium cuningtonii	·	·	✓	·
Peridinium lomnickii	✓	✓	·	·
Peridinium umbonatum	✓	·	·	·
Chrysophyta				
Dinobryon divergens	·	✓	✓	·

Table 2 (continued)

TAXA	TA	TB	TC	TŢ
Dinobryon sertularia	·	·	✓	·
Dinobryon sociale	·	·	✓	·
Mallomonas tonsurata	·	✓	·	·
Bacillaryophyta				
Achnanthes biosoletiana	✓	✓	·	·
Achnanthes lanceolata	·	✓	✓	✓
Achnanthes minutissima	✓	✓	✓	✓
Amphora libyca	·	✓	✓	✓
Amphora pediculus	·	·	·	✓
Anomoeoneis sphaerophora	·	·	✓	·
Asterionella formosa	✓	✓	✓	·
Caloneis silicula	·	·	·	✓
Cocconeis pediculus	✓	·	✓	·
Cocconeis placentula	✓	·	✓	·
Cyclostephanos dubius	·	✓	·	✓
Cyclotella menenghiniana	·	✓	✓	✓
Cyclotella pseudostelligera	·	✓	✓	✓
Cymatopleura solea	·	✓	✓	✓
Cymbella aspera	✓	✓	✓	✓
Cymbella cistula	·	✓	✓	✓
Cymbella cuspidata	·	·	✓	·
Cymbella gracilis	✓	·	·	·
Cymbella mesiana	✓	·	·	·
Cymbella minuta	✓	✓	·	·
Denticula kuetzingii	·	·	✓	·
Ephitemia adnata	✓	✓	✓	✓
Ephitemia sorex	·	✓	✓	✓
Eunotia bilunaris	✓	·	✓	·
Fragilaria capucina	✓	✓	✓	✓
Fragilaria construens	·	✓	·	✓
Fragilaria crotonensis	·	✓	✓	✓
Fragilaria pinnata	·	✓	·	·
Fragilaria ulna	✓	✓	✓	✓
Frustulia vulgaris	·	✓	·	·
Gomphonema angustum	✓	·	✓	·
Gomphonema acuminatum	·	·	·	✓
Gomphonema parvulum	✓	✓	✓	✓
Gomphonema truncatum	✓	✓	✓	✓
Gyrosigma spencerii	·	✓	✓	✓
Hantzschia amphioxys	·	·	·	✓
Melosira varians	·	✓	✓	✓
Meridion circulare	·	·	·	✓
Navicula bacillum	·	✓	✓	·
Navicula capitata	·	·	·	✓

Table 2 (continued)

TAXA	TA	TB	TC	TȚ
Navicula cincta	✓	✓	✓	✓
Navicula cryptocephala	·	✓	·	✓
Navicula cuspidata	·	·	✓	✓
Navicula elginensis	·	✓	·	✓
Navicula lanceolata	·	·	✓	·
Navicula radiosa	·	·	✓	·
Navicula viridula	·	✓	·	·
Neidium alpinum	·	·	·	✓
Nitzschia amphibia	·	✓	✓	✓
Nitzschia fruticosa	✓	·	·	·
Nitzschia palea	·	✓	✓	✓
Nitszschia sinuata var. *tabellaria*	✓	·	·	✓
Pinnularia borealis	·	·	·	✓
Pinnularia gibba	·	·	✓	✓
Pinnularia subcapitata	·	✓	·	·
Rhopalodia gibba	·	✓	✓	✓
Stauroneis phaenicarteron	·	·	✓	·
Tabellaria fenestrata	✓	·	✓	·
Chlorophyta				
Ankistrodesmus bibraianus	·	·	✓	·
Ankistrodesmus fusiformis	✓	·	·	·
Ankistrodesmus gracilis	·	·	✓	·
Botryococcus braunii	✓	✓	✓	✓
Chlamydomonas incerta	·	·	✓	·
Closterium acutum	✓	✓	·	✓
Closterium aciculare	·	·	✓	·
Closterium gracile	·	·	✓	·
Cosmarium inconspicuum	·	✓	·	·
Crucigenia fenestrata	✓	·	✓	·
Dictyosphaerium pulchellum	✓	✓	✓	·
Dictyosphaerium subsolitarium	·	✓	·	·
Dictyosphaerium tetrachotomum	·	✓	·	·
Eresmophaera viridis	✓	·	·	·
Eudorina elegans	✓	·	·	·
Eutetramorus globosus	·	✓	·	·
Franceia ovalis	✓	·	·	·
Gonium pectorale	·	·	✓	·
Keratococcus mucicola	·	✓	·	·
Kirchneriella lunaris	✓	·	·	·
Kirchneriella obesa	✓	·	✓	·
Micractinium pusillum	·	✓	·	·
Monoraphidium contortum	✓	✓	·	·
Mougeotia sp.	✓	·	✓	·
Oocystis borgei	✓	·	·	·

Table 2 (continued)

TAXA	TA	TB	TC	TŢ
Oocystis marsonii	✓	·	·	·
Oocystis solitaria	✓	·	·	·
Pandorina morum	·	·	·	✓
Pediastrum tetras	·	·	·	✓
Radiococcus planctonicus	·	✓	·	·
Scenedesmus acuminatus	·	✓	·	·
Scenedesmus opoliensis	·	✓	·	·
Scenedesmus quadricauda	·	✓	·	·
Selenastrum bibraianum	·	·	✓	·
Selenastrum capricornutum	·	✓	·	·
Selenastrum gracile	·	·	✓	·
Spirogyra sp.	✓	·	✓	·
Staurastrum boreale	·	·	✓	·
Staurastrum lunatum	✓	·	·	·
Staurastrum manfeldtii	·	·	✓	✓
Staurastrum paradoxum	·	·	✓	·
Staurastrum tetracerum	✓	✓	✓	✓
Staurastrum quadrangulare	✓	·	✓	·
Staurodesmus dejectus	✓	·	·	·
Tetraedron limneticum	·	✓	·	·
Tetraedron minimum	✓	·	✓	✓
Tetrastrum triangulare	✓	·	·	·
Zygnema sp.	✓	·	✓	·
TOTAL TAXA	**54**	**66**	**70**	**52**

Bacillariophyta (58) and Chlorophyta (48). Diatoms (Bacillariophyta) recorded the highest percentage in three sampling sites (Fig. 2), followed by green algae (Chlorophyta) and blue-green algae (Cyanoprokariota). From these three phylla, true planktonic elements were identified (*Asterionella formosa, Cyclotella meneghiniana*, *C. pseudostelligera, Fragilaria* sp., *Tabellaria fenestrata*, *Ankistrodesmus* sp., *Chlamydomonas* sp., *Oocystis* sp., *Scenedesmus*, *Aphanizomenon flos*-aquae, *Microcystis aeruginosa* etc.), but also numerous benthic species, that entered the planktonic samples due to the sampling method (*Achnanthes* sp., *Amphora* sp., *Cymbella* sp., *Gomphonema* sp., *Closterium* sp., *Monoraphidium* sp, some species of *Oscillatoria*). This hierarchy of the three dominant algal phylla was constant for Tăul Brazi, Tăul Cornii, and Tăul Ţarinii. For Tăul Anghel however, green algae recorded the highest percentage, and not diatoms (Fig. 2), while Euglenophyta and Chrysophyta were absent. Diatoms reached the highest percentage in Tăul Ţarinii, where Euglenophyta and Dinophyta recorded a low number of species and Chrysophyta was again absent. These differences could be explained by local ecological conditions that vary from one lake to another: the extent of the riparian zone, land use around the lake, "water blooms", human impacts etc.

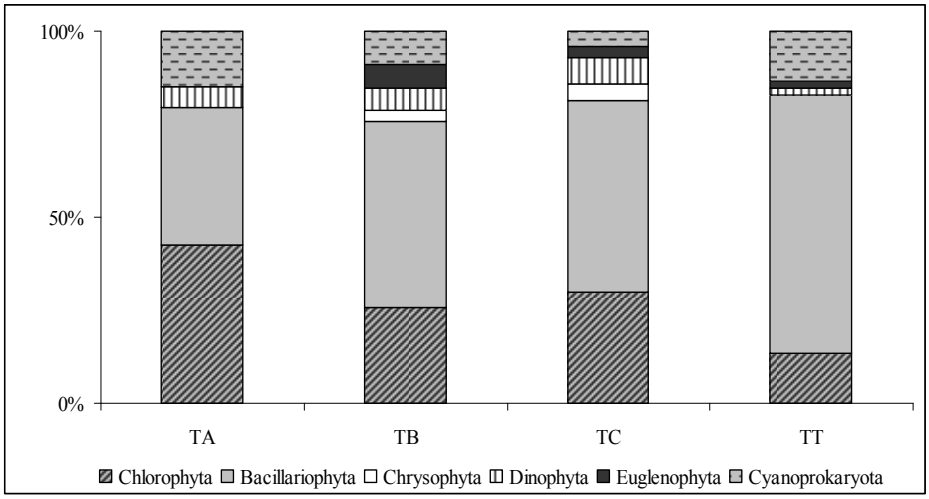

Fig. 2. The percentage of the major phytoplanktonic groups in the four lakes sampled in August 2010 (**TA** - Tăul Anghel; **TB** - Tăul Brazi; **TC -** Tăul Cornii; **TŢ** - Tăul Ţarinii)

Planktonic microcrustacean communities. Twenty-three species were identified in the five lakes from the Roşia Montană region, out of which 12 were cladocerans and 11 copepods (Table 3). Most of the cladoceran individuals were parthenogenetic females, with or without eggs, because of the dominance of the asexual reproduction during favorable seasons, summer in particular. Only one male belonging to *Disparalona rostrata* was found in Tăul Brazi, in November 2009, showing the appearance of the sexual reproduction in the population. In case of copepods, both females and males were identified, together with copepodites and nauplii (Table 3).

Most of the microcrustaceans found in the five lakes considered for the present study were cosmopolitan (*Chydorus sphaericus, Simocephalus vetulus* or *Mesocyclops leuckarti*). Many were characteristic to the pelagic areas of aquatic ecosystems, like *Daphnia galeata*, or known to be part of the neuston, like *Scapholeberis kingi*. However, species preferring benthic habitats were also found (*Ectocyclops phaleratus*), together with elements living in regions dominated by submerged macrophytes (*Pleuroxus* sp.), because the samples were collected near the banks of the lakes. The highest number of taxa (copepodites and nauplii not included) was identified in Tăul Brazi. However, this situation cannot reflect a higher diversity compared to the communities from the other four lakes. In fact, this large number of species is caused by the fact that microcrustaceans collected in the benthic samples from July 2009, November 2009 and April 2010 were also analyzed, while in Tăul Anghel, Tăul Cornii and Tăul Ţarinii only samples taken in August 2010 were considered.

Table 3.

List of planktonic microcrustacean species, including copepodites and nauplii, identified in the five lakes from Roşia Montană region (TA - Tăul Anghel; TB - Tăul Brazi; TC - Tăul Cornii; TM - Tăul Mare; TŢ - Tăul Ţarinii; ✓ - present; · - not present)

TAXA	TA	TB	TC	TM	TŢ
Cladocerans					
Alona guttata	·	✓	·	·	·
Alona rectangula	✓	·	✓	·	✓
Bosmina longirostris	·	✓	·	·	✓
Chydorus sphaericus	·	✓	✓	·	·
Daphnia galeata	·	·	·	·	✓
Disparalona rostrata	·	✓	·	·	·
Ilyocryptus agilis	·	✓	·	·	·
Macrothrix laticornis	·	✓	·	·	·
Pleuroxus aduncus	✓	✓	·	·	·
Pleuroxus truncatus	✓	✓	·	·	·
Scapholeberis kingi	·	✓	✓	·	·
Simocephalus vetulus	✓	·	·	·	·
Copepods					
Attheyella crassa	·	✓	·	·	·
Bryocamptus minutus	·	✓	·	·	·
Cyclops vicinus	·	✓	·	·	·
Ectocyclops phaleratus	·	✓	✓	·	·
Eucyclops macruroides	·	·	·	✓	·
Eucyclops serrulatus proximus	·	✓	·	✓	·
Macrocyclops albidus	·	✓	·	✓	·
Mesocyclops leuckarti	·	✓	✓	✓	✓
Microcyclops varicans	·	✓	·	·	·
Paracyclops fimbriatus	·	·	·	✓	·
Thermocyclops oithonoides	✓	✓	✓	·	·
copepodites	✓	✓	✓	✓	✓
nauplii	✓	✓	✓	✓	✓
TOTAL TAXA	**5**	**18**	**7**	**5**	**4**

The relative percentage abundance was calculated only for the samples collected in August 2010 from Tăul Anghel, Tăul Brazi, Tăul Cornii and Tăul Ţarinii (Fig. 3). Copepodites and nauplii recorded the highest percentage abundance in all four lakes. Adults of *Thermocyclops oithonoides* recorded lower percentage in Tăul Anghel and Tăul Cornii, while in Tăul Ţarinii the microcrustaceans had more balanced abundance values. The most abundant species were euplanktonic or characteristic to areas rich in macrophytes (Fig. 3).

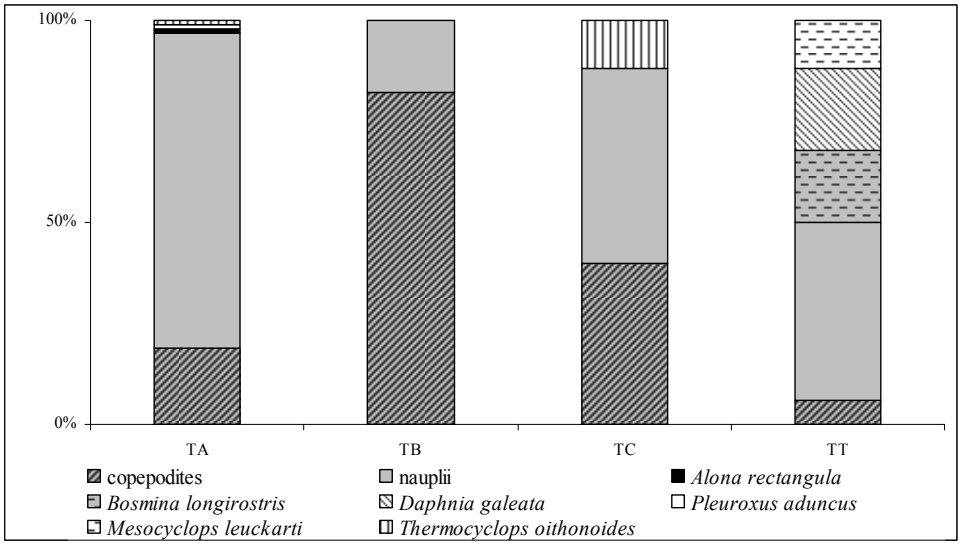

Fig. 3. The relative percentage abundance of microcrustacean species identified in the four lakes sampled in August 2010 (**TA** - Tăul Anghel; **TB** - Tăul Brazi; **TC** - Tăul Cornii; **TŢ** - Tăul Ţarinii).

Benthic invertebrate communities. A high diversity characterized the two lakes where benthic samples were taken (Tăul Brazi and Tăul Mare): twenty aquatic invertebrate groups were identified in the sampled seasons. The lowest number of taxa was recorded in spring in Tăul Brazi (10) and in Tăul Mare (8), while the highest number was found in autumn (14 and 15, respectively) (Table 4).

Aquatic earthworms (Oligochaeta) are a numerous group in benthic habitats, well represented in the two water bodies from Roşia Montană. Mayflies (Ephemeroptera), that includes populations intolerant to pollution, were represented by two species: *Cloeon dipterum* and *Caenis horaria*. The first one lives in still, clear waters, with many submerged macrophytes (Belfiore, 1983). It reached a higher abundance in Tăul Mare, being present in all samples, except for the spring one taken from Tăul Brazi. On the other hand, *Caenis horaria* recorded a higher number of individuals, especially in Tăul Brazi.

Compared to the previous study carried out in 2006 by Roşia Montană Gold Corporation, 13 families and genera were added to the taxa list for Tăul Mare, and 11 families and genera for Tăul Brazi (Table 5). Insect larvae belonging to Ephemeroptera, Odonata, Trichoptera and Coleoptera were not found in the first study.

Table 4.

The presence of benthic invertebrate taxa in the considered lakes and seasons from Roşia Montană region (TB - Tăul Brazi; TM - Tăul Mare; ✓ - present; - not present)

TAXA	TB Apr. 2010	TB Jul. 2009	TB Nov. 2009	TM Apr. 2010	TM Jul. 2009	TM Nov. 2009
Oligochaeta						
Naididae	✓	✓	✓	·	✓	✓
Tubificidae	✓	✓	✓	✓	✓	✓
Hydrachnidia						
Arrenurus sp.	✓	·	·	·	·	·
Crustacea						
Ostracoda	✓	✓	✓	·	✓	✓
Collembola	·	✓	✓	·	·	✓
Coleoptera						
Gyrinus sp.	·	✓	·	·	✓	✓
Agabus sp.	·	✓	✓	·	·	·
Diptera						
Ceratopogonidae	✓	✓	✓	✓	✓	✓
Chironomidae	✓	✓	✓	✓	✓	✓
Ephydridae	·	·	✓	·	·	·
Psychodidae	·	·	·	·	·	✓
Stratiomidae	·	·	·	·	✓	✓
Ephemeroptera						
Cloeon dipterum	·	✓	✓	✓	✓	✓
Caenis horaria	✓	✓	✓	✓	·	✓
Odonata						
Coenagrion sp.	·	·	✓	·	·	✓
Erythroma viridis	·	·	·	·	✓	·
Trichoptera						
Apatania sp.	✓	·	·	·	·	✓
Bivalva	·	·	·	✓	✓	·
Gastropoda						
Ancylus sp.	·	·	✓	·	·	·
Nematoda	✓	✓	✓	✓	✓	✓
Tardigrada	✓	✓	✓	✓	✓	✓
TOTAL TAXA	**10**	**12**	**14**	**8**	**12**	**15**

Table 5.

Comparison between the present work and the study made by Roşia Montană Gold Corporation - RMGC (TB - Tăul Brazi; TM- Tăul Mare; ✓ - present; - not present)

TAXA	**TB-**RMGC study	**TB-**present study	**TM-**RMGC study	**TM-**present study
Oligochaeta				
Naididae	✓	✓	·	✓
Tubificidae	✓	✓	✓	✓
Hydrachnidia				
Arrenurus sp.	·	✓	·	·
Crustacea				
Ostracoda	·	✓	·	✓
Collembola	✓	·	✓	✓
Coleoptera				
Gyrinus sp.	·	✓	·	✓
Agabus sp.	·	✓	·	·
Megaloptera				
Sialidae	✓	·	·	·
Diptera				
Ceratopogonidae	✓	✓	✓	✓
Chironomidae	✓	✓	✓	✓
Ephydridae	·	✓	·	·
Psychodidae	·	·	·	✓
Stratiomidae	·	·	·	✓
Ephemeroptera				
Cloeon dipterum	·	✓	·	✓
Caenis horaria	·	✓	·	✓
Odonata				
Coenagrion sp.	·	✓	·	✓
Erythroma viridis	·	·	·	✓
Trichoptera				
Apatania sp.	·	✓	·	✓
Bivalva	·	·	·	✓
Gastropoda				
Ancylus sp.	·	✓	·	·
Nematoda	✓	✓	·	✓
Heteroptera				
Pleidae	✓	·	·	·
Tardigrada	·	✓	·	✓
TOTAL TAXA	**8**	**16**	**4**	**17**

High values of percentage abundance were recorded by Oligochaeta and Chironomidae (Fig. 4). In April 2010, the highest abundances were reached by Oligochaeta in both lakes, followed by Nematoidea. The percentage of Chironomidae recorded in spring 2010 is higher in Tăul Mare compared to Tăul Brazi (13.01% compared to 1.77%). In summer, both Oligochaeta and Chironomidae represented the dominant groups in the two lakes. Ostracoda reached 12.61% in Tăul Mare, while Ephemeroptera was better represented in Tăul Brazi (with 13.3%). Oligochaeta continued to be the dominant group, reaching similar percentages in Tăul Brazi (55.79%) and Tăul Mare (57.23%). Chironomidae reached the second place as percentage abundance in both lakes, while Nematoidea recorded a higher percentage in Tăul Brazi (Fig. 4).

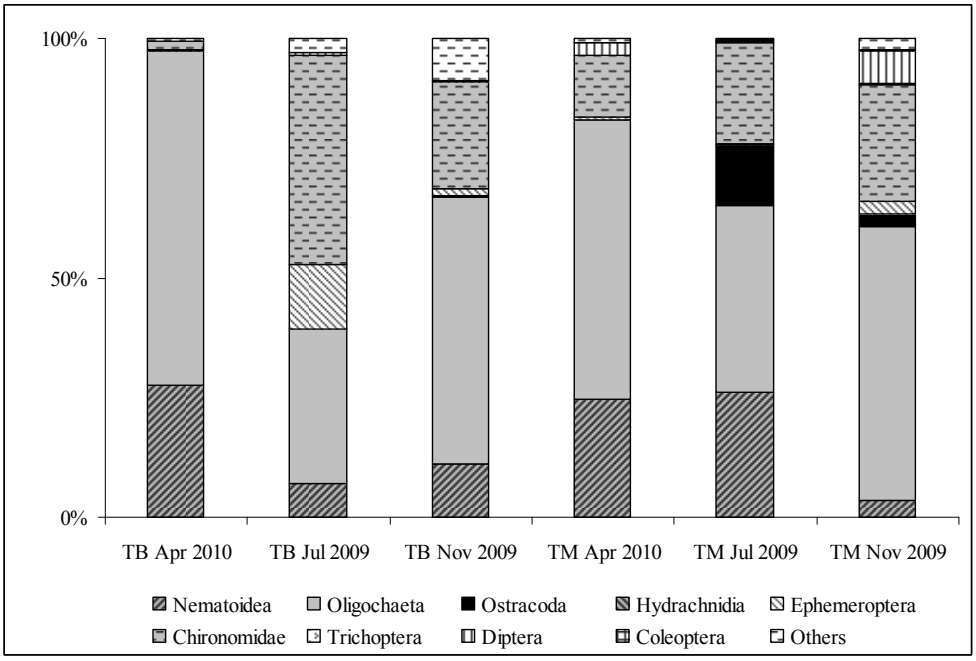

Fig. 4. The percentage abundance (%) of benthic invertebrate groups from Tăul Brazi (TB) and Tăul Mare (TM) in three different seasons

The assessment of the ecological status was possible only in four lakes, where phytoplankton and microcrustacean samples were collected in August 2010: Tăul Anghel, Tăul Brazi, Tăul Cornii and Tăul Ţarinii. The indices according to Oltean (1977) were calculated only for Tăul Brazi Tăul Cornii and Tăul Ţarinii. In the first two lakes, "water blooms" caused by *Peridinium* sp. and *Ceratium* sp. (Dinophyta) were recorded, while *Arthrospira jenneri* (Cyanoprokariota) induced

"water blooms" in Tăul Ţarinii. The values of these indices indicated eutrophic waters (beta-eutrophy index: 0.53 and 0.56 in Tăul Brazi and Tăul Cornii; zeta-eutrophy index: 34.64 in Tăul Ţarinii). The other eutrophy indices calculated according the qualitative composition of algal communities (Willén, 2000) also showed eutrophic conditions, different lakes being in different stages of eutrophy, depending on the local ecological conditions (Table 6).

In case of saprobic level assessment based on indicator values of certain algal species (Sládeček, 1973), most of the phytoplankton taxa were characteristic for xenosaprobic, oligosaprobic and beta-mesosaprobic conditions, showing a fairly good water quality from the point of view of the quantity of decomposing organic matter in the system. However, there were also elements indicating critical saprobic levels (beta- alfa-mesosaprobic) and extremely high concentrations of decomposing organic matter (alfa-mesosaprobic to polisaprobic). These species characteristic for poor water conditions represented 13% from all species with indicator values in Tăul Anghel, 17% in Tăul Brazi, 24% in Tăul Cornii and 32% in Tăul Ţarinii. These results are supported by the organic pollution indices, calculated at the species level (I_S) and at the genus level (I_G) (according to Willén, 2000). Their values suggested low organic pollution in Tăul Anghel and Tăul Brazi, moderate organic pollution in Tăul Cornii and high organic pollution in Tăul Ţarinii (Table 6).

Table 6.

The values of various indices based on planktonic algal communities (TA - Tăul Anghel; TB - Tăul Brazi; TC - Tăul Cornii; TŢ - Tăul Ţarinii)

INDICES	TA	TB	TC	TŢ
1. Nygaard compound index	8.33	9.66	5.00	3.01
2. Thunmark index (Chlorophyta)	4.25	4.00	1.28	1.02
3. Heinoen trophic index	14.00	18.00	9.00	12.00
4. Organic pollution index I_S	12	13	15	19
5. Organic pollution index I_G	10	14	15	16

Nineteen microcrustacean species are recorded with indicator values for saprobic levels of the water bodies. Only one species, *Ectocyclops phaleratus*, is known to indicate higher quantities of decomposing organic matter (beta-alfa-mesosaprobic conditions). Eighteen cladoceran or copepod species were characteristic for oligosaprobic, oligo-beta-mesosaprobic or beta-mesosaprobic waters, indicating a moderately good quality of the lakes.

A general estimation of the ecological status for Tăul Brazi and Tăul Mare could be made based on benthic invertebrate communities. Even if the dominant groups are those tolerant to pollution (Oligochaeta and Chironomidae), the high number of taxa (16 and 17, respectively), together with the presence of sensitive species from Ephemeroptera and Odonata groups can indicate that the two lakes are characterized by a moderate - good water quality.

Conclusions

1. A high number of taxa was identified in case of all three biotic communities considered for the present study: 145 phytoplankton taxa, 23 microcrustacean species and 20 benthic invertebrate groups.
2. Based on phytoplankton and microcrustacean communities, the four lakes sampled in August 2010 (Tăul Anghel, Tăul Brazi, Tăul Cornii and Tăul Ţarinii) are characterized by relatively good water quality as concerns the quantity of decomposing organic matter. However, all four lakes are eutrophic.
3. The increased diversity and the composition of the benthic invertebrate community analyzed in Tăul Brazi and Tăul Mare might also show a fairly good general condition for these two lakes.

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SPATIAL AND TEMPORAL VARIATIONS OF ENZYMATIC ACTIVITY IN BIOFILMS OCCURRING INTO A DRINKING WATER TREATMENT PLANT IN CLUJ, ROMANIA

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SUMMARY: Drinking water quality is affected by the structure and physiology of the attached microbial communities in drinking water treatment, storage and distribution. In order to characterise microbial biofilms which occur during the drinking water treatment process, the present study aims to evaluate the spatial and temporal variability in enzymatic activity. Biofilm samples from clarification and from rapid sand filtration steps were analyzed in February, April, August and November 2011. Dehydrogenase, phosphatase and catalase enzymes were targeted. Heterotrophic bacteria were also quantified in biofilms, while raw water physical, chemical and microbiological parameters were monitored. High rates of phosphatase, catalase and dehydrogenase activities were recorded in biofilms developed in the clarification step. Increased numbers of sessile heterotrophic bacteria were found during spring and summer. The enhanced enzymatic activity registered during the cold season may be attributed to extracellular enzymes accumulation into the biofilm matrix. Also, the physiologically stressed state of bacteria, caused by drinking water treatment procedures may induce an intensive enzymatic activity. No significant difference was noticed in the intensity of enzymatic activities within biofilms developed on concrete and steel surfaces. However, higher catalase activity was registered on steel surfaces, where the accumulation of corrosion products and tuberculation were observed. A reduction in enzymatic rates throughout water purification process was recorded.

Keywords: catalase, dehydrogenase, drinking water biofilm, phosphatase, microbial activity.

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Introduction

Microbial activity is one of the most important factors in water purification processes (Hendel *et al*., 2001). The quality of tap water is therefore dependent on the structure and physiology of the microbial communities in drinking water treatment, storage and transportation.

The main strategy of bacterial growth in aquatic systems is represented by the attached heterogenic communities (Costerton, 1994). Biofilms play an essential barrier role in biological water treatment through the entrapment of particulate material (including microbial pathogens) as well as through nutrient removal (Fonseca *et al*., 2001, Långmark *et al*., 2004; LeChevallier and Au, 2004; Tellen *et al*., 2010). Bacterial communities associated in biofilms display enhanced biodegradation and bioaccumulation characteristics, when compared to planktonic cells. On the other hand, occurrence of biofilms in storage and distribution systems may induce water quality deterioration (O'Connor and O'Connor, 2001; Skraber *et al*., 2005; Lee *et al*., 2006; Wingender and Flemming, 2011) and biocorrosion (Videla and Characklis, 1992; Beech and Flemming, 2000; Coetser and Cloete, 2005). The main sources of deposits in drinking water treatment and distribution systems are particulate matter transported by water, microbial activity and physicochemical reactions both at the water/walls interface and within the water bulk (Echverría, 2009).

Inorganic and organic nutrients in water and sediments are biochemically recycled by microbial enzymes. In biofilms, where most of the microbial biomass is concentrated, these immobilized enzymes have great importance (Miettinen *et al*., 1996). They may be used as an indicator of microbial diversity, offering an overview of environmental change, but not an absolute measurement of any specific group of organisms (Carpa, 2011).

Bacteria are the primary colonizers of surfaces submerged in water. They produce a wide range of enzymes, broadly categorized as ectoenzymes (associated with the cell, but expressed outside the cytoplasmatic membrane) and extracellular enzymes (present as free forms). Their release into the environment provides a basis for the interaction between bacteria and substrates, resulting in effects such as biocorrosion (Beech *et al*., 2005).

In order to characterize the microbial communities in drinking water associated biofilms, the present study aims to evaluate the spatial and temporal variability in enzymatic activity. Dehydrogenase, phosphatase and catalase enzymes were targeted. Microbial loads in biofilms were also quantified, while raw water physical, chemical and microbiological parameters were monitored.

Biofilms activity may be explored by assessing intercellular dehydrogenase levels rather than viable biomass (Fonseca *et al*., 2001). The overall dehydrogenase rates depend on the activities of various dehydrogenases, which are a fundamental part of the enzyme system of all microorganisms (enzymes of the respiratory metabolism, the citrate cycle and nitrogen metabolism), being considered a good measure of microbiological redox systems (von Mersi and Schinner, 1991).

Extracellular phosphatases are involved in the microbially mediated degradation of organic matter and in the regeneration of inorganic nutrients from macromolecular compounds (Jones, 1997; Hendel *et al*., 2001).

Catalases belong to the enzyme group of oxidoreductases that have a very high potential in environmental protection, their expression in microorganisms functioning as a response to exogenous stressors (Polek, 2008).

High extracellular enzyme activities reflect high substrate availability and typically are characteristic of untreated water (Emtiazi *et al*., 2004). Thus, decrements in enzymatic processes in biofilms throughout drinking water treatment are to be expected. Numerous studies assessing enzymatic activities in soils, sediments and aquatic ecosystems are available, with regard to environmental pollution. Less is known about the enzymatic potential of biofilms developed during industrial processes, such as those associated with drinking water treatment.

Materials and Methods

Sampling

Drinking water treatment plant (DWTP) of Cluj County, Romania (coordinates 46°44'N latitude and 23°22'E longitude) is fed from three alternative sources: Tarniţa, Someşul Cald and Gilău lakes. The waterfall system of dam reservoirs is situated in Western Romania. Drinking water is obtained by a conventional treatment process, involving microstraining, prechlorination, coagulation-flocculation, rapid sand filtration and final disinfection by chlorination.

Biofilm samples from concrete and steel walls of a clarifier as well as from a sand filter were collected and analyzed, in February, April, August and November 2011. At the same moment, raw water entering the DWTP was sampled and analyzed.

In order to capture representative biofilm samples for analysis, a sampling protocol was designed. Biofilms were collected from the preset areas of the concrete and steel walls of a clarifier. Sand grains from a filter operating more than 30 hours were sampled from three areas. Amounts of 100 g biofilms and 500 g sand beads were homogenized and aliquots were used for analyses.

Biofilm analyses

Enzymatic activity and microbial growth were assessed in biofilms. All the procedures were performed in sterile conditions. The enzymatic activities were assessed according to methods described by Drăgan-Bularda (2000), after 48 hours of incubation at 37°C for dehydrogenase and phosphatase, respectively after two hours for catalase.

The levels of actual dehydrogenase were assessed by 2, 3, 5-triphenil tetrazolium chloride (TTC) reduction to triphenyl formazan (TPF). Red formazan was extracted in alcohol-acetone solution and measured at 485 nm using the Jasco V-530 UV-VIS spectrophotometer. Dehydrogenase activity was was expressed as mg TPF per gram of biofilm.

The hydrolytic dissociation of phenyl phosphate disodium substrate was evaluated in phosphatase activity assessment. The phenol products resulted were coloured with Gibbs reagent. Blue indophenol concentration was measured spectrophotometrically at 600nm. Phosphatase activity was expressed in mg phenol per gram of biofilm.

Catalase activity was investigated targeting hydrogen peroxide decomposition, and was expressed in mg split H_2O_2 per gram of biofilm.

The enzymatic potential of biofilms developed on concrete, steel and sand surfaces through drinking water treatment was assessed based on the enzymatic indicators of biofilm quality (EIBQ) (Muntean *et al*., 1996).

For microbiological analyses, biofilm samples were suspended in tryptone water and vortexed with glass beads. Serial dilutions up to 10^{-6} were prepared for inoculation. The total numbers of viable mesophilic bacteria were assessed by heterotrophic plate counts (HPC) method, inoculating the sample in yeast extract agar medium. Plates were incubated at 37°C for 48 hours and at 22°C for 72 hours (SR EN ISO 6222/2004).

Raw water analyses

Physical, chemical and microbiological parameters of raw water were measured: temperature, pH, alkalinity, hardness, conductivity, dissolved oxygen, biochemical oxygen demand (BOD), chemical oxygen demand (COD), nitrates, chlorides, sulphates, phosphates, aluminium, iron, calcium, magnesium, HPC at 37°C and HPC at 22°C. Water temperature, pH and conductivity were measured with calibrated laboratory equipments. Alkalinity, hardness, dissolved oxygen, BOD, COD, chlorides, calcium and magnesium contents were determined by titration. Nitrates, sulphates and phosphates were quantified using the Lambda 40Bio Perkin Elmer spectrophotometer. Aluminium and iron concentrations were ascertained using the atomic absorption spectrophotometer contrAA700, Analytik Jena.

Statistics

Data were analyzed using descriptive and inferential statistics. Correlation, regression and statistical significance testing in parameter pairs were performed (Ţigan *et al*., 2001), using the Microsoft Office Excel 2007 software.

Results and discussions

Enzymatic activity in biofilms

The levels displayed by the enzymatic activities targeted reveal intense microbial processes in biofilms at the moment of the analyses and also previously, due to their accumulation within the exopolymeric matrix.

The average, minimum, maximum values and standard deviations for the three quantitative enzymatic activities in drinking water associated biofilms are presented in Table 1. Dehydrogenase activity recorded the highest levels on concrete substratum (an annual average of 1.424 mg TPF/g), while catalase and phosphatase activities were more intense on steel-associated biofilms (23.602 mg split H_2O_2/g, respectively 1.916 mg phenol/g). Lower levels in enzymatic activities were registered in the sand filter.

Table 1. Quantitative enzymatic activities in drinking water associated biofilms.
DHA = dehydrogenase activity; PHA = phosphatase activity;
CAT = catalase activity; C = concrete; S = steel; F = filter sand.

Enzymatic activity	Sample origin	Average	Minimum activity	Maximum activity	Standard deviation
DHA (mg TPF/g)	C	1.424	1.236	1.628	0.161
	S	1.242	1.100	1.357	0.120
	F	0.014	0.005	0.018	0.006
PHA (mg phenol/g)	C	1.891	0.918	3.456	1.091
	S	1.916	1.077	3.456	1.053
	F	0.193	0.054	0.480	0.194
CAT (mg split H_2O_2/g)	C	19.125	13.147	23.687	4.381
	S	23.602	19.947	27.993	4.149
	F	4.448	3.400	5.327	0.875

A seasonal variability occurred, with maximum values registered during the cold season (Fig. 1). Decrements in enzymatic processes in biofilms throughout drinking water treatment were recorded.

The observed difference between enzymatic activities registered in clarifier's biofilms compared with those in the sand filter may be a consequence of the lower content of biomass per gram of sand beads.

Dehydrogenase activity indicates the potential of microbial community to support biochemical processes, influenced by various environmental factors (Shengnan *et al*., 2011). Raw water characteristics and treatment steps are the key factors influencing microbial activity in bulk water and also in the attached biofilms.

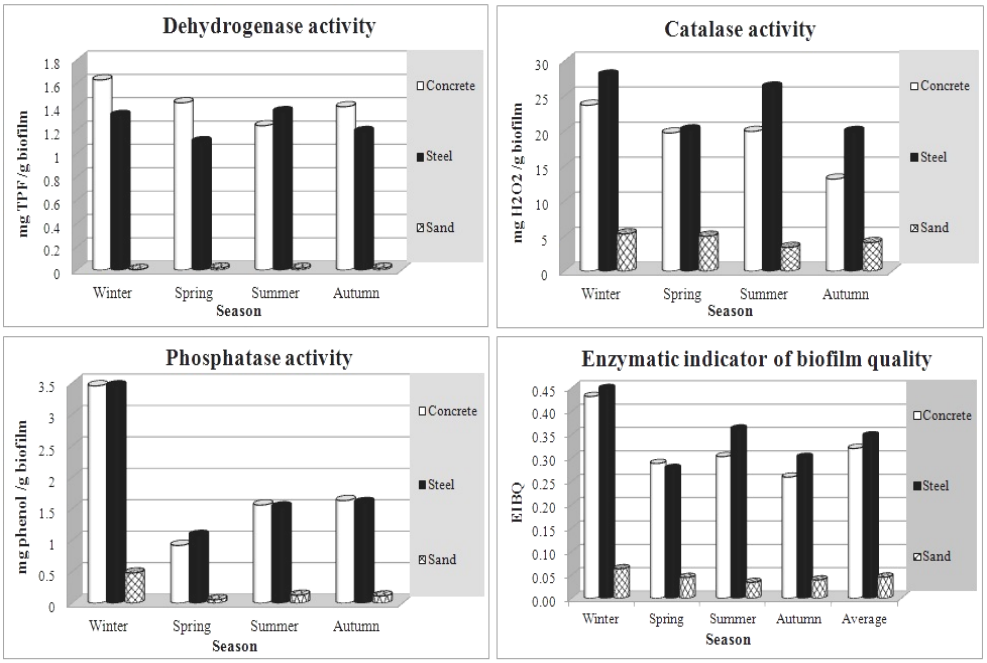

Figure 1. Enzymatic activity in biofilms: dehydrogenase, phosphatase, catalase activities and the enzymatic indicator of biofilm quality.

Enhanced phosphatase activity during the cold season was also found in lake water samples, in contrast to the samples from the ground water, where the highest phosphatase rates were registered during the summer (Miettinen *et al*., 1996). Phosphatases catalyze the liberation of orthophosphate from organic phosphorus compounds (Jansson *et al*., 1988), contributing in mineralization processes.

Bacterial catalases are, in general, intracellular enzymes. The induction of an extracellular form of catalase represents a response to oxidative stress, dependent on the bacteria phase of growth (Busalmen *et al*., 2002).

Pronounced differences in dehydrogenase and phosphatase levels were observed between biofilms from the clarifier and those from the sand filter. Catalase activity still recorded high rates in sand filter biofilms. This may suggest that drinking water treatment procedures led to a stressful environment for the attached bacterial consortia. Bacteria are not only exposed to changing environments in their natural habitats but also during industrial processing. Activation of stress adaptation mechanisms can provide cell robustness to harsher conditions (den Besten *et al*., 2010). The measurement of antioxidant enzymes such as catalase activity in biofilms is an estimation of the capacity of the whole community to respond to oxidative stress (Bonnineau *et al*., 2010), e.g. disinfection procedures. The antimicrobial effect of gaseous chlorine used in water purification encounter antimicrobial effects based

on its products of dissociation, hypoclorous acid (HOCl) and hypoclorite ions (OCl^-). They are extremely reactive with numerous components of the bacterial cell and act as oxidants (LeChevallier and Au, 2004).

The enzymatic activities recorded the highest values during the cold season, corresponding to the lowest microbial loads in both biofilm and water. It may be a consequence of extracellular enzymes accumulation within the biofilm matrix and also to the entry of bacterial populations into a physiologically stressed state. The presence of dehydrogenase activity denotes the presence of living microorganisms at the sampling moment, while phosphatase and catalase persist longer in biofilms and sediments (Carpa and Butiuc-Keul, 2009). The latter enzymes may originate in lysate cells and persist due to their accumulation in organic-mineral colloids (Paşca *et al*., 1993). Within a biofilm matrix, bacterial enzymes may exhibit depolarizing effects for months, even in the absence of viable cells (Beech, 2003).

In source water sediments, the maximum rates in both enzymatic activity and in heterotrophic plate counts were registered during autumn (Curticăpean and Drăgan-Bularda, 2007a; 2007b). Such peak events were considered to be a consequence of the higher concentrations of nutrients and of warm water. Other studies investigating the free-living bacteria and the attached consortia in river biofilms (Böckelmann *et al*., 2000; Araya *et al*., 2003) reported lower levels of microbial activity from summer to winter. Authors attributed this phenomenon to the entry of bacterial populations into a physiologically stressed stated with low levels of enzymatic activity (i.e. injured or in a dormant state) or to a reduction in respiratory rates. Bacterial respiratory metabolism, assessed in the present study by measuring dehydrogenase activity, indicates increments during the winter season. Therefore, the results suggest that the lower rates in multiplication and metabolism associated with a drop in water temperature may lead to enhanced respiratory rates. Growth of cells entering the stationery phase may cause an accumulation of endogenous oxidants, resulting in an increase in respiratory activity (Polek, 2008).

The enzymatic indicator of biofilm quality (EIBQ) based on dehydrogenase, phosphatase and catalase activities ranges from 0.449 to 0.034. EIBQ rates reveal the highest enzymatic potential of drinking water associated biofilms during the winter, in the clarifier (Fig. 1). Average EIBQ values of 0.348 registered in steel-associated biofilms, 0.321 in concrete-developed biofilms and 0.045 in sand filter's biofilms.

Microbial growth in biofilms

Microbiological analyses revealed high numbers of cultivable bacteria (Table 2). Maximum HPC values were recorded by incubation at 22°C (1.6×10^7 bacteria per gram of biofilm), as characteristic for environmental samples.

Microbial loads of more than 10^{10} cells per gram were previously found in different types of deposits formed within water systems (Characklis, 1988; Barbeau *et al*., 2005; Rubulis *et al*., 2008).

Table 2. Microbial loads in drinking water associated biofilms. C = concrete; S = steel; F = filter sand.

Heterotrophic bacteria	Sample origin	Average	Minimum HPC	Maximum HPC	Standard deviation
HPC 37°C (CFU/g)	C	2382234	429090	4227273	1796552
	S	2553167	420900	5000000	1888636
	F	6272	250	12273	5288
HPC 22°C (CFU/g)	C	8340462	1724500	16363636	6118530
	S	9829280	3827300	15909091	5917087
	F	97411	23636	152550	54402

An increased microbial activity registered on steel-associated biofilms, closely followed by consortia developed on concrete substrata (Fig. 2).

A difference of three orders magnitude may be observed in colony counts of clarifier's biofilms compared with those from the sand filter. It may be attributed to the lower content of biomass at the surface of sand grains.

In terms of seasonal evolution, peaks in microbial loads were observed during spring and summer, corresponding to the seasonal variation of planktonic microbiota (Farkas *et al*., 2011).

The attached microbial communities probably act as biological buffers against increases in organic matter and nutrients in bulk water (Hendel *et al*., 2001). Heterotrophic bacteria are the key players in the process of organic matter recycling, by decomposition and mineralization within aquatic environments (Muntean, 2007; Cunha *et al*., 2010).

To restrict microbial growth, biofilm removal and disinfection procedures are applied.

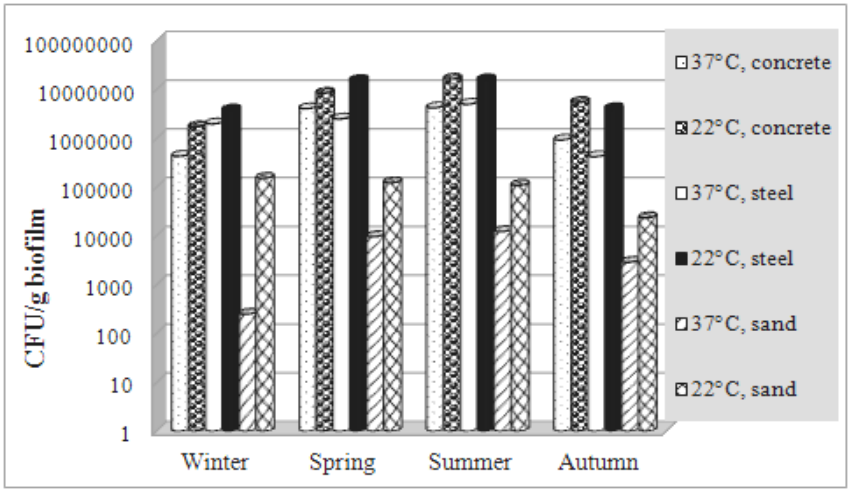

Figure 2. Heterotrophic plate counts in biofilm.

Raw water characteristics

Physico-chemical and microbiological parameters of raw water were monitored once with biofilms (Table 3). High quality water as regard of physical and chemical parameters is feeding the DWTP, as indicated also by previous studies (Farkas *et al*., 2011). Microbial load in bulk water slightly increased during summer and autumn. Previous investigations in the quality of water sources and of the raw water entering the treatment plant revealed seasonal variations and correlations, as characteristic of freshwater environments in the temperate area (Farkas *et al*., 2010; 2011). Planktonic bacteria recorded loads with four to six orders of magnitude lower than the attached heterotrophs.

Table 3. Raw water parameters monitoring during the four seasons in 2011.

Parameter	Measuring unit	Winter	Spring	Summer	Autumn
Temperature	°C	3.60	4.90	13.50	10.10
pH	pH units	7.13	7.22	7.17	7.43
Alkalinity	mEq/L	0.66	0.58	0.70	0.66
Hardness	°dH	2.49	2.08	2.17	2.86
Conductivity	µS/cm	93.70	86.80	86.00	118.40
Dissolved oxygen	mg/L	11.90	12.54	10.28	9.18
BOD	mg/L	2.38	1.37	2.04	1.15
COD	mg/L	2.56	2.19	2.07	1.94
Nitrates	mg/L	2.55	2.58	2.17	1.88
Chlorides	mg/L	2.52	2.36	1.81	1.68
Sulphates	mg/L	6.82	6.18	8.01	5.57
Phosphates	mg/L	1.88	2.16	0.02	0.22
Aluminium	µg/L	142.60	131.70	54.21	26.54
Iron	mg/L	0.080	0.086	0.025	0.050
Calcium	mg/L	12.22	11.30	11.71	15.23
Magnesium	mg/L	3.40	2.17	2.29	3.16
HPC at 37°C	CFU/mL	10	15	22	27
HPC at 22°C	CFU/mL	85	260	101	304

Factors influencing the enzymatic activity in biofilms

Considering that the attached microbial consortia were developed within an artificial environment, during drinking water treatment process, multiple factors are expected to influence their activity. Raw water parameters, biofilm characteristics, surface materials and treatment steps are variables influencing enzymatic activity in the attached consortia.

Very strong positive correlations ($r > 0.781$) with high levels of significance ($p < 0.01$) were found between all enzymatic activities, with each other and with EIBQ (Table 4). Dehydrogenase and catalase activities were also positively correlated with heterotrophic bacteria in biofilms ($p < 0.05$). Correlations between EIBQ and HPC, PHA with HPC and DHA with HPC at 22°C were not statistically significant ($p > 0.05$).

In freshwaters, soils and sediments, strong positive correlations were found between bacterial abundance and enzyme activities, and between enzyme activities and organic matter content or temperature (Fonseca *et al*., 2001; Taylor *et al*., 2002; Araya *et al*., 2003; Curticăpean and Drăgan-Bularda, 2007b). Other authors did not found a clear response to nutrient availability or a strong correlation to bacterial growth (Cunha *et al*., 2010). Limited amounts of nutrients were assumed to induce enzymes production in bacteria, in order to liberate them from organic matter (Harder and Dijkhuizen, 1983).

Table 4. Pearson correlations in DWTP biofilms: * $p < 0.05$; ** $p < 0.01$

Microbial activity	DHA	PHA	CA	EIBQ	HPC 37°C
PHA	0.781**				
CA	0.906**	0.818**			
EIBQ	0.944**	0.906**	0.976**		
HPC 37°C	0.600*	0.212	0.662*	0.550	
HPC 22°C	0.547	0.162	0.611*	0.503	0.890**

Bacterial populations living attached to surfaces adapt to nutritional conditions offered by the bulk water (Miettinen *et al*., 1996). The limited number of events in present monitoring did not allow a correlation analysis between microbial activities in biofilms and raw water parameters. Despite the high quality of the raw water processed (Table 3), tremendous numbers of heterotrophic bacteria are harboured in the associated biofilms. Phosphorus availability was previously found to limit microbial growth in soils (Kunito *et al*., 2012) and biofilms formation in drinking water systems (Lehtola *et al*., 2002). Peaks of phosphorus concentration in raw water were reached during spring, corresponding to an ascending trend of microbial growth both in water and biofilms.

The present study found the highest microbial loads both in biofilms and in raw water during the warm season, but maximum levels of extracellular enzymes during the winter. This endorses the hypothesis that bacterial enzymes may have accumulated within the biofilm matrix.

Assessing the relationship between the substrate material and the enzymatic activity in the attached biofilms, no statistical significant differences ($p > 0.05$) were observed between concrete and steel-associated consortia. When comparing the enzymatic rates in biofilms from the clarifier with those in the sand filter, significant differences ($p < 0.05$) have been observed (Table 5).

Table 5. p-values of t-test for enzymatic activities in DWTP biofilms

Parameter	p-value
DHA concrete- DHA sand	0.0004
DHA steel- DHA sand	0.0002
PHA steel- PHA sand	0.0485
CA concrete- CA sand	0.0071
CA steel- CA sand	0.0028

Student's test results suggest that microbial communities developed in the clarifier evolved in the same sense, independently on the substrate material. On the other hand, the attached microbiota in drinking water associated biofilms was influenced by the water treatment procedures, since enzymatic activity in both biofilms (concrete and steel), developed in the settling step significantly differ from that in the filtration stage.

Similar findings were observed in the occurrence of certain species of microorganisms or physiological groups of bacteria, compared in the three types of biofilms (Farkas *et al*., *in press*).

Effects of enzymatic activity in drinking water associated biofilms

Enzymatic activity in drinking water associated biofilms may encounter beneficial but also detrimental effects. Biofilms may be exploited in water purification process, for their enhanced mineralization, biodegradation and bioaccumulation properties. On the other hand, microbial activity may generate biocorrosion. Subsequent biofilm accumulation, together with corrosion products, may result in infrastructure deterioration and water quality degradation.

Extracellular enzymes were previously found to affect the composition and properties of exopolymeric matrix, thereby influencing biofilm development (Tielen *et al*., 2010). In the current study, increased levels of phosphatase, catalase and dehydrogenase activities were recorded in biofilms, especially during winter. Clarification process allows an enhanced deposition and biofilm formation, comparing to the rapid sand filtration. Higher enzymatic activity rates were found within biofilms occurring in the settling step. The reduction in enzyme activities during the purification process from surface water to drinking water reflects an improvement in its trophic status (Emtiazi *et al*., 2004).

In the present situation, raw water chemical quality, characterized by low levels of nutrients, may not indicate the requirement for an additional biological treatment step. Nevertheless, the increased enzymatic activity in biofilms indicates an enhanced mineralization process within the attached biofilms. Previous studies reported faecal contamination in source waters (Lumperdeanu and Drăgan-Bularda, 2002; Curticăpean and Drăgan-Bularda, 2007b; Farkas *et al*., 2010; 2011; Muntean *et al*., 2010) and the presence of opportunistic pathogens in biofilms developed in

through drinking water processing (Farkas *et al*., *in press*). Bio-sand filtration might be thus suggested as suitable for microbial removal (LeChevallier and Au, 2004; de Vet *et al*., 2009; Tellen *et al.*, 2010).

Active enzymes within the biofilm matrix together with metal ions bound by bacterial extracellular polymeric substances can catalyze cathodic reactions, contributing to the biocorrosion process (Beech *et al*., 2005). Microbially influenced corrosion does not only occur when bacteria are present on metallic materials. Extracellular enzymes accumulated within the biofilm matrix may induce surface deterioration. Even if some bacteria responsible for corrosion are already well-known, the enzymes which may have a key role in biocorrosion are not fully identified (Landoulsi *et al*., 2008). Catalase represents a bacterial catalyst for the oxygen reduction process, which involves electron recycling during the enzymatic decomposition of peroxide (Busalmen *et al*., 2002). Therefore, the more intense catalase activity recorded in steel-associated biofilms may be explained. The accumulation of corrosion products and tuberculation were observed on the surface of steel materials throughout drinking water treatment.

Conclusions

The enzymatic activities assessed in current study provide a direct insight into the spatial and temporal distribution of microbial processes within a drinking water treatment plant.

High rates of phosphatase, catalase and dehydrogenase activities were recorded in drinking water associated biofilms, mostly in the clarification step. Increased numbers of heterotrophic bacteria were found to reside in biofilms during spring and summer. Thus, the enhanced enzymatic activity during the winter season may be a result of extracellular enzymes accumulation into the biofilm matrix. Also, the physiologically stressed state of bacteria due to the drinking water treatment processes may induce an intensive enzymatic activity.

There were no significant differences in the abundance of enzymatic activities within biofilms developed on concrete and steel surfaces. A reduction in enzymatic rates throughout water purification process was observed.

Statistical significant positive correlations were found between all enzymatic activities, with each other, with EIBQ and with heterotrophic bacteria in biofilms.

In terms of biocorrosion, enhanced catalase activity on steel surfaces may contribute in the accumulation of corrosion products and tuberculation.

The beneficial effects of enzymatic activity in drinking water biofilms consisting in mineralization and biodegradation properties are to be further considered.

In the present situation, the raw water of high chemical quality may not indicate the requirement for an additional biological treatment step. Bio-sand filtration might be suggested as suitable for microbial removal. As the international authorities recommend, biofilms surveillance and control through drinking water treatment, storage and distribution is advisable.

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==ERRATUM==

INHIBITION OF MYELOPEROXIDASE, BUT NOT SUPEROXIDE DISMUTASE ACTIVITY SHOWS PROTECTIVE FUNCTION IN THE EX VIVO MODEL OF BULLOUS PEMPHIGOID

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